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Contribution to the management of gastrointestinal tumors in dogs and cats

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List of abbreviations

AC	Adenocarcinoma
AFP	Alpha-fetoprotein
AL	Alimentary lymphoma
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AUC	Area under the curve
BSC	Body condition score
CBC	Complete blood count
CDH1	E-cadherin-encoding gene
CEA	Carcinoembryonic antigen
CG	Contrast gastrography
CRP	C-reactive protein
CT	Computed tomography
CTC	Computed tomography colonography
CTE	Computed tomography enterography
CTEc	Computed tomography enteroclysis
CV	Coefficient of variation
DCG	Double-contrast gastrography
DV	Dorsoventral
E-cad	E-cadherin
EGFR	Epidermal growth factor receptor
EUS	Endoscopic ultrasonography or endosonography
EGC	Early gastric cancer
HER	Human epidermal receptor
^{18}FDG	Fludeoxyglucose F 18
FISH	Fluorescent in situ hybrisation
FNA	Fine needle aspiration
GC	Gastric cancer
GEJ	Gastro-oesophageal junction
GI	Gastrointestinal
GIST	Gastrointestinal stromal tumor

HHCT	Helical hydro-computed tomography
HHM	Humoral hypercalcemia of malignancy
HMGB1	High-mobility group B1 proteins
IBD	Inflammatory bowel disease
IHC	Immunohystochemistry
LDH	Lactate dehydrogenase
LL	Left lateral
LR	Likelihood ratio
MALT	Mucosa associated lymphoid tissue
MPR	Multiplanar reconstruction
MRE	Magnetic resonance enterography
MRI	Magnetic resonance imaging
NPV	Negative predictive value
PET/CT	Positron emission tomography/computed tomography
^{18}FDG-PET/CT	Positron emission tomography with fludeoxyglucose F 18
PG	Pneumogastrography
PPV	Positive predictive value
PTHrP	Parathyroid hormone-related peptide
ROC	Receiver operating characteristics
RL	Right lateral
SAA	Serum amyloid A
SC	Serum chemistry
SE	Standard error
sTK	Serum thymidine kinase activity
TK1	Thymidine kinase 1
TNF-α	Cytokine tumor necrosis factor-α
TNM	Tumour node metastasis
xURL	Fold of upper reference limit
US	Ultrasonography
VD	Ventrodorsal

CHAPTER I

GENERAL INTRODUCTION

Diagnostic imaging and endoscopic finding in dogs and cats with gastrointestinal tumors: a review

Adapted from:

Terragni R, Vignoli M, van Bree HJ, Gaschen L, Saunders JH. Diagnostic imaging and endoscopic findings in dogs and cats: a review. Schweiz Arch Tierheilkd 2014;156:569-76.

1. Anatomy of the stomach and intestines

The stomach (ventriculus, gaster)

Curvatures and surfaces of the stomach

The stomach possesses visceral and parietal surfaces, and greater and lesser curvatures. The **visceral surface** (*facies visceralis*) presents a convex outer surface that faces mainly dorsally, but also caudodextrally. The **parietal surface** (*facies parietalis*) faces to the left and cranially as well as ventrally. The **greater curvature** (*curvatura ventriculi major*) forms the convex border of the stomach. The **lesser curvature** (*curvatura ventriculi minor*) forms the concave border of the stomach.

Regions of the Stomach

The **cardiac part** (*pars cardiaca*) of the stomach is the portion that blends with the esophagus. The opening into the stomach is the *ostium cardiacum.*

The **fundus of the stomach** (*fundus ventriculi*) is the rather large blind outpocketing located to the left and dorsal to the cardia. On the left the **cardiac notch** (*incisura cardiaca*) is formed between the cardia and the bulging fundic part.

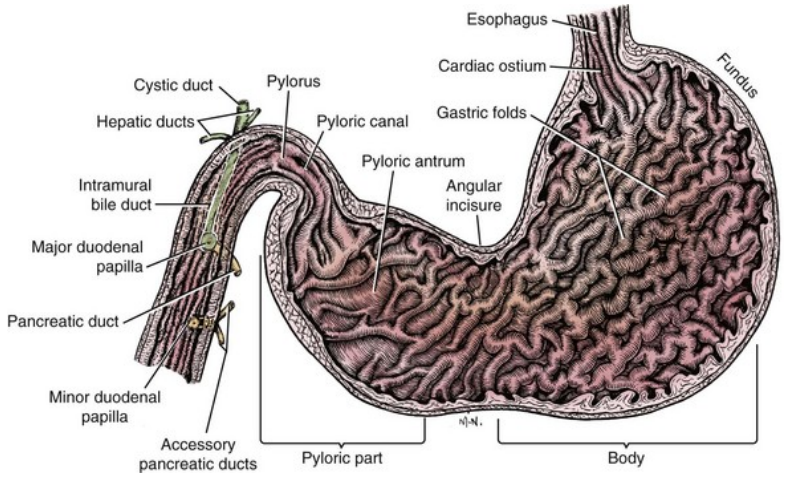

Fig 1. Longitudinal section of the stomach and proximal portion of the duodenum. (From Evans HE, de Lahunta A: Miller's anatomy of the dog, ed 4, St Louis, 2013, Saunders/Elsevier.)

The **body of the stomach** (*corpus ventriculi*) is the large middle portion of the organ. It extends from the fundus on the left to the pyloric part on the right. The **pyloric part** (*pars pylorica*) is approximately the distal third of the stomach as measured along the lesser curvature from the angular notch to the duodenum. The initial two-thirds is thin-walled and expanded to form the **pyloric antrum** (*antrum pyloricum*). The distal third is contracted and bent. The **pylorus** (*pylorus*) is largely surrounded by a thick, double muscular sphincter at the entrance to the duodenum. This forms the narrowest part of the cavity of the stomach. The lumen of the pylorus is the *ostium pyloricum*. The **pyloric canal** (*canalis pyloricus*) is the short narrow segment between the pyloric antrum and the pylorus.

Coats of the Stomach

The **serous coat** (*tunica serosa*) completely covers the stomach except for an extremely narrow line on the distal half of the greater curvature. The serosa of the stomach is extremely thin and elastic. It adheres closely to the stomach musculature by a scanty amount of subserous connective tissue, the *tela subserosa.*

The **muscular coat** (*tunica muscularis*) of the stomach consists essentially of an outer longitudinal and an inner circular layer of smooth muscle fibers. The outer **longitudinal layer** (*stratum longitudinale*) is continuous with the essentially outer longitudinal layers of both the duodenum and the esophagus. As the longitudinal fibers cover the pyloric portion they are particularly thick on the sides between the curvatures. The inner **circular layer** (*stratum circulare*) of the stomach is more complete and specialized than is the longitudinal layer. At the cardia the circular layer is thickened to form the small **cardiac sphincter** (*m. sphincter cardiae*). Surrounding the pyloric canal, the inner circular layer is well developed. The pylorus, which opens into the duodenum, is also surrounded by a circular muscle termed the **pyloric sphincter** (*m. sphincter pylori*). The **submucous coat** (*tela submucosa*) consists of a dense but thin elastic layer of areolar tissue that more firmly attaches to the mucosa than to the muscularis. It contains the finer branches of the gastric vessels and nerves. The **mucous coat** (*tunica mucosa*) in carnivores is entirely glandular. It consists of a columnar surface epithelium, a glandular lamina propria, and a lamina muscularis mucosae consisting of muscular fibers which may be irregularly interwoven or. The normal color of the mucosa in the body and fundus of a fresh stomach is pink to

grayish red. In the pyloric region it is lighter in color. The color varies with the amount of contained blood, as well as the freshness of the material.

Glands of the Stomach

The glands of the stomach are known as the **gastric glands** (*glandulae gastricae*). They are branched tubular glands with necks and bodies that reach nearly to the lamina muscularis
mucosae. Three types of gastric glands are recognized in the dog. These are the cardiac glands, the gastric glands proper, and the pyloric glands. The **cardiac glands** (*glandulae cardiacae*), are found in a narrow zone around the cardia.

The **gastric glands proper** (*glandulae gastricae [propriae]*), or fundic glands, occupy approximately two thirds of the gastric mucosa. The **pyloric glands** (*glandulae pyloricae*) are found in the pyloric part of the stomach. The difference between the various gland zones is in the type of cells they contain, and therefore in the nature of the secretion they produce. The glands of the cardiac and pyloric regions function mainly to produce mucus; the proper gastric glands produce hydrochloric acid indirectly and the enzyme pepsin.

As in most other areas of the alimentary canal, lymph nodules are scattered throughout the mucosa of the stomach. Some of these extend through the lamina muscularis mucosae into the submucosa.

The intestines

Small intestine

The **small intestine** (*intestinum tenue*) extends from the pylorus of the stomach to the ileocolic orifice leading into the large intestine. The small intestine consists of three main parts, the relatively fixed and short proximal loop, or **duodenum**; the freely movable, long, middle and distal portions, the **jejunum**; and the very short terminal part, the **ileum**.

Duodenum

The duodenum is the first and most fixed part of the small intestine. For descriptive purposes the duodenum is divided into four portions and two flexures. The **cranial portion** (*pars cranialis*) of the duodenum is short between the pylorus and cranial

duodenal flexure. It arises from the pylorus and almost immediately turns acutely to the right and caudally as the **cranial duodenal flexure** (*flexura duodeni cranialis*) that runs essentially caudally. The **descending portion** (*pars descendens*) of the duodenum, approximately 15 cm long, runs caudally from the cranial portion nearly to the pelvic inlet. The **caudal duodenal flexure** (*flexura dudeni caudalis*) is between the descending and transverse parts of the duodenum. The **transverse portion** (*pars transversa [pars caudalis]*) of the duodenum connects the descending and ascending portions from right to left and is approximately 5 cm long. The **ascending portion** (*pars ascendens*) of the duodenum runs obliquely cranially and to the left from the transverse part. Then it makes a sweeping curve ventrally to form the **duodenojejunal flexure** (*flexura duodenojejunalis*). At this flexure the jejunum continues the duodenum ventrally, caudally, and to the left, and enters into the formation of numerous coils and kinks (festoons), which constitute most of the intestinal mass.

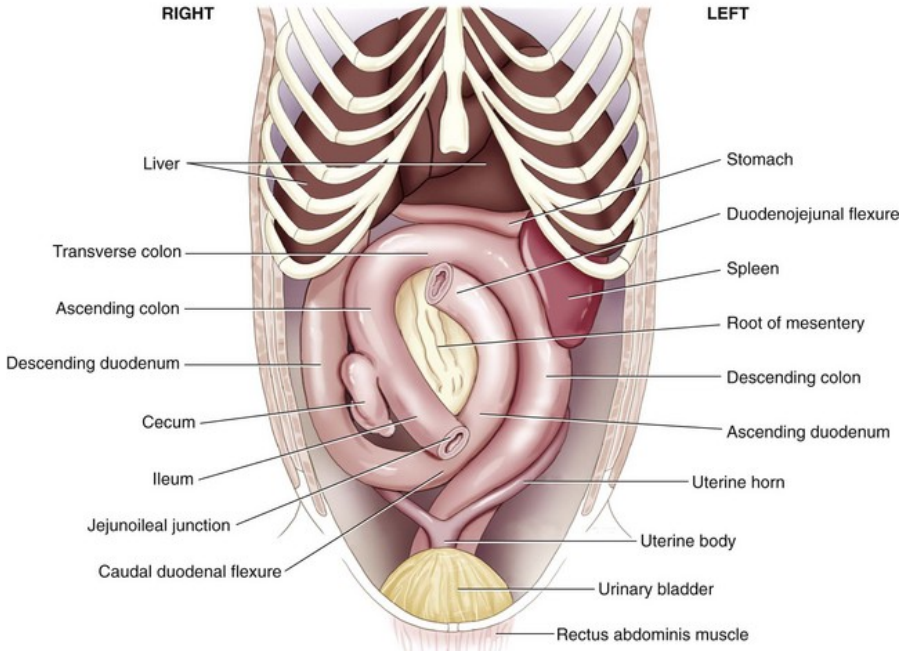

Fig 2. Intestines of a cat: Removal of the jejunum reveals the duodenum, which begins at the pylorus and continues caudally as the descending duodenum along the right side of the abdomen, where it is in contact with the parietal peritoneum. The descending duodenum runs nearly to the pelvic inlet, where it turns, forming the caudal duodenal flexure moving from right to left. Then it continues cranially as the ascending duodenum. (From Smith BJ: Gastrointestinal organs and spleen. In Canine anatomy, Philadelphia, 1999, Lippincott Williams & Wilkins.)

Jejunum and Ileum

The jejunum and ileum compose the remainder and the majority of the small intestine with the jejunum being the longest portion. The jejunum begins at the duodenojejunal flexure, and the ileum ends by opening into the initial portion of the ascending colon as the **ileal papilla** (*papilla ilealis*) with an **ileal orifice** (*ostium ileale*) and an associated circular **sphincteric muscle** (*m. sphincter ilex*). The ileum of the dog is approximately the last 15 cm of the small intestine.

Coats of the small intestine

The small intestine, like the other parts of the alimentary tract, is composed of mucous, submucous, muscular, and serous tunics.

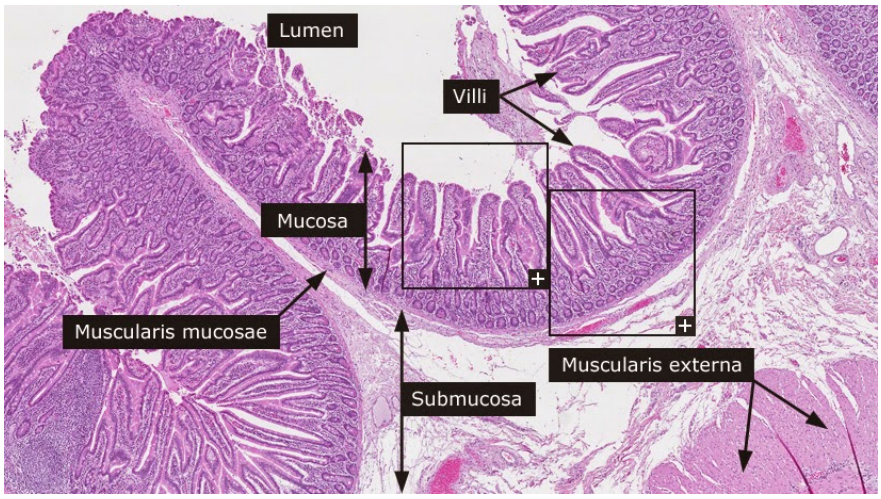

Fig 3. The histologic layers of the small intestine of a cat.

The **mucous coat** (*tunica mucosa*), throughout the small intestine of the dog, presents a free surface that is velvety due to the presence of innumerable **intestinal villi** (*villi intestinales*). The single-layered epithelial surface cells are of two types. One type consists of the columnar cells, which function is absorption, and the other type consists of the goblet, mucusproducing cells. The deeper part of the mucosa is occupied largely by the **intestinal glands** (*glandulae intestinales*) and diffuse lymphoid tissue and single nodules. In approximately 22 areas throughout the small intestine of the dog the lymphoid nodules are grouped together to form the **aggregated lymph nodules** (*nodull lymphatici aggregati*). The *lamina muscularis*

mucosae, was found to be three times thicker in the dog than it was on average in the other domestic animals studied. It is finally divided into inner circular and outer longitudinal layers. The **submucous coat** (*tela submucosa*) resembles that of the stomach and large intestine. It loosely binds together the mucous and muscular layers. The smaller blood vessels, lymphatics, and the submucous nerve plexus are located in it. The **muscular coat** (*tunica muscularis*) consists of a relatively thin outer longitudinal layer (*stratum longitudinale*) and a thicker inner circular layer (*stratum circulare*). The **serous coat** (*tunica serosa*) of the small intestine is composed of the peritoneum, which almost completely covers the duodenum.

Large intestine

The **large intestine** (*intestinum crassum*) is short and unspecialized. In general, it is a simple tube, only slightly larger in diameter than the small intestine and is divided into cecum, colon, rectum, and anal canal. It begins at the ileal papilla and ends at the anus.

Cecum

The **cecum** exists as a diverticulum of the proximal portion of the colon, the ascending colon. It is approximately 5 cm long and 2 cm in diameter at its colic end.

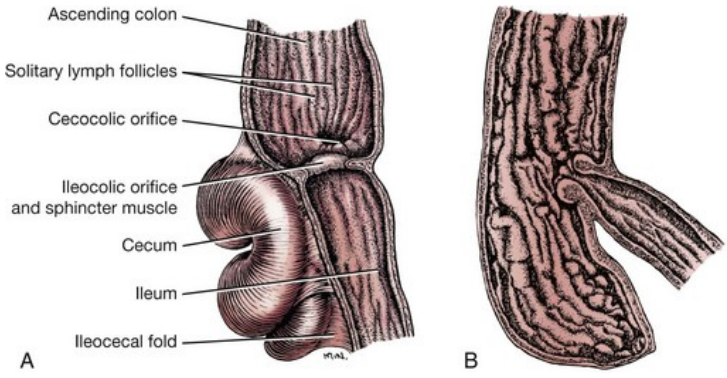

Fig 4. Longitudinal section through ileocolic orifice of the canine ileocolic junction, ventral aspect. **B,** The feline cecum is a short, conelike structure with no compartmentalization and rarely contains gas. (**A** from Evans HE, de Lahunta A: Miller's anatomy of the dog, ed 4, St Louis, 2013, Saunders/Elsevier. **B** from Thrall DE, editor: Textbook of veterinary diagnostic radiology, ed 5, Philadelphia, 2007, Saunders/Elsevier. Reprinted from O'Brien TR: Radiographic diagnosis of abdominal disorders in the dog and cat, Davis, CA, 1981, Covell Park Veterinary.)

It irregularly tapers to the rather blunt **apex** (*apex ceci*), which is less than 1 cm in diameter and usually points caudoventrally. The large middle portion of the organ may be referred to as the **body** (*corpus ceci*). The only communication of the cecum is with the beginning of the ascending colon by means of the **cecocolic orifice** (*ostium cecocolicum*).

This opening lies approximately 1 cm from the ileocolic orifice. The **cecal sphincter** (*m. sphincter ceci*) is a specialization of the inner circular muscular coat that guards the cecocolic orifice. At the apical end of the body beyond its attachment to the ileum extends the single or double **ileocecal fold** (*plica ileocecalis*.

Colon

The **colon** is divided into ascending, transverse, and descending portions and their connecting flexures. The **ascending colon** (*colon ascendens*) begins at the ileal ostium, runs cranially, and ends at the right-angled right colic flexure. It usually is approximately 5 cm long, but this varies greatly. The **transverse colon** (*colon transversum*) forms an arc that runs from right to left cranial to the root of the mesentery. It is approximately 7 cm long. The **descending colon** (*colon descendens*) is the longest segment of the colon. It extends from the left colic flexure to a transverse plane passing through the pelvic inlet, where it is continued by the rectum without demarcation. It is approximately 12 cm long and usually quite straight.

Rectum

The **rectum** begins at the pelvic inlet, where it is continuous cranially with the descending colon; it ends ventral to the second or third caudal vertebra, at the beginning of the anal canal. It is straight, approximately 5 cm long, and 3 cm in diameter.

The most prominent feature of the rectal mucosa is the presence of approximately 100 **solitary lymph nodules** (*lymphonoduli solitarii*). These nodules are each approximately 3 mm in diameter and 1 mm high. The free surface of each is umbilicated, forming a crater, or rectal pit.

Coats of the Large Intestine

Except for the terminal part, all of the large intestine has the usual four coats as found in the small intestine. These are the mucous, submucous, muscular, and serous tunics, or coats.

The **mucous coat** (*tunica mucosa*) contains numerous solitary lymph nodules that are most numerous in the rectum. The mucosa of the large intestine contains many folds and depending on the type of contraction, these plicae are either longitudinal or circular. The **intestinal glands** (*glandulae intestinales*) of the large intestine are longer, straighter, and richer in goblet cells than are those of the small intestine. They are lined by a columnar epithelium. The *lamina muscularis mucosae* consists of muscle fibers that are poorly arranged in two strata. The **submucous coat** (*tela submucosa*) does not differ appreciably from that of the small intestine. Many of the solitary lymph nodules are located partly within it. This tunic contains the submucous nerve plexuses and many vessels in the meshes of loose connective tissue. The **muscular coat** (*tunica muscularis*) is uniform in thickness. The *stratum longitudinale* is not concentrated in muscular bands (taenia). The *stratum circulare* of the large intestine resembles that of the small intestine, except that it is thicker. Its caudal portion forms the **internal anal sphincter muscle**. The **serous coat** (*tunica serosa*) resembles that of the small intestine. It covers the colon, cecum, and much of the rectum, as the visceral peritoneum. The anal canal and the caudal portion of the rectum are retroperitoneal.

2. Gastrointestinal tumors in dogs and cats

Gastric tumors are uncommon in dogs and cats representing less than 1% of all malignancies.[1] The mean age of affected animals is 8 years (15 years for leiomyomas), with a 2:1 male-to-female ratio.[2] A higher incidence of gastric carcinoma has been reported in Belgian shepherd dogs, Collies, Staffordshire bull terriers and Siamese cats, implying a strong genetic component.[3,4] The low prevalence of the disease is more influenced by breed popularity in the different countries and may have resulted in an underestimation of the incidence in the uncommon breeds.[5] A strong environmental component has been suggested in dogs because experimental long-term administration of nitrosamines may induce gastric carcinoma.[6] Several epidemiological studies in humans support a positive association between gastric cancer risk and nitrite and nitrosamine. Humans are exposed to a wide range of

nitrosamines and nitrosamides from diet (cured meat products, dried salted fish, smoked preserved food, pickled and salty preserved food), tobacco smoking, work place and drinking water. Certain dietary characteristics, including high sodium intake or low product (vegetables/fruit) consumption are recognized risk factors for human GC. Many studies have also examined genetic changes related to GC development and progression in people. While in humans Helicobacter pylori is an influential factor in the carcinogenesis of gastric tumors, causing an epithelium damage, association has not been documented in dogs and cats.[7]

Patients with gastric tumors usually present themselves with a history of progressive vomiting, hematemesis, melena, anorexia and weight loss. Chronic vomiting is caused by neoplastic stenosis, gastric ulceration and/or gastric motility disorders. Weight loss may be a result of anorexia, malabsorption and maldigestion, loss of protein and blood from an ulcer, and/or generalized tumor cachexia. Occult blood in the feces may be detected. The clinical signs can last from weeks to months.[7] Laboratory abnormalities may include panhypoproteinemia, microcytic hypochromic anemia related to chronic gastrointestinal blood loss and malabsorption, elevated liver enzymes due to hepatic metastasis or obstruction of the common bile duct.[7]

Both leiomyoma and leiomyosarcoma may present with paraneoplastic hypoglycemia[8],

The majority of canine gastric tumors are malignant and epithelial in origin and affect the distal two-thirds of the stomach. Adenocarcinoma (AC) accounts for 70–80% of canine stomach tumors.[9] They often appear scirrhous (firm, white serosa) and have been termed linitis plastica (leather bottle) because of their firm, nondistensible texture. In adenocarcinoma, lesions can be diffusely infiltrative and expansile, often with a central crater and ulceration (Fig. 5), or may look more polypoid.[10] The prognosis in cases of gastric carcinoma is poor. Furthermore, 70–90% of gastric carcinomas have metastasized by the time of diagnosis and underwent euthanasia.[5] The most common sites reported for metastases are the regional lymph nodes, with others being the omentum, duodenum, liver, pancreas, spleen, oesophagus, adrenal glands, and lungs.[5]

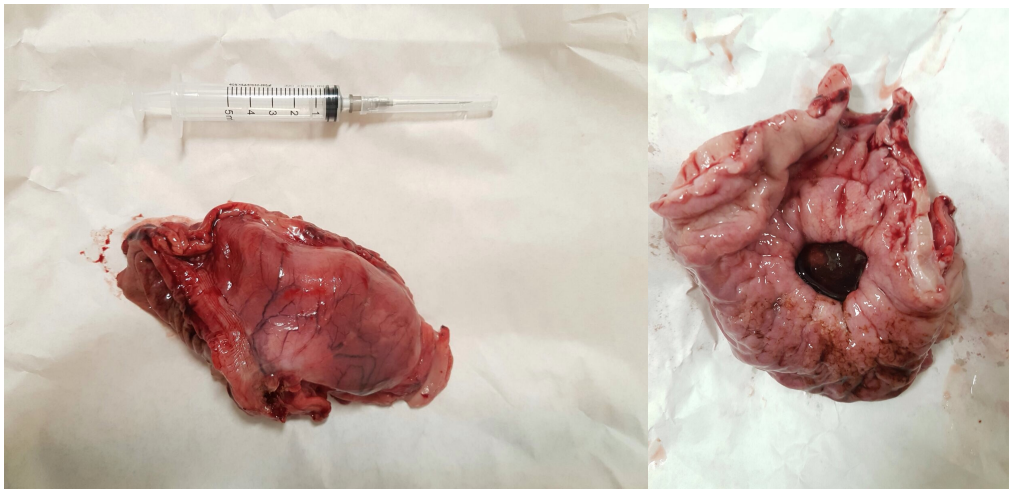

Fig 5. Resected AC of the stomach in a Golden Retriever. The tumour was lying close to the pyloric sphincter. Note the large, perforating ulcer (right image).

Leiomyosarcoma is often bulky, tends to affect the entire thickness of the gastric wall and most commonly occurs at the pyloric antrum.

Lymphoma is the most common gastrointestinal (GI) neoplasm in the cat and may be solitary in the stomach or one component of a systemic involvement. In the post-feline leukaemia virus era, the total incidence of feline leukaemia virus-related lymphoma has decreased but there has been an increase in the incidence of non-feline leukaemia virus lymphoma, particularly in the alimentary tract.[11] Lymphocytic-plasmacytic enterocolitis has been suggested as a precursor to feline intestinal lymphoma, but this suggestion has not been supported by other studies. Dietary allergies and chronic inflammatory bowel disease have been postulated as precursors to intestinal lymphoma but associations have not been documented.[11]

Other malignancies include mast cell tumor (mastocytoma), extramedullary plasmacytoma, fibrosarcoma[7] and, very rarely, gastric carcinoid. Metastases may reach the stomach by lymphatic or blood vessels. Benign stomach tumors are much less common. Typically, gastric adenomas and leiomyomas are solitary incidental findings located near the cardia.[8] A diffuse extension and/ or malignant transformation is rare, but possible. Final diagnosis has to be made with histopathology.

Intestinal tumors in dogs and cats represent less than 10% of all malignancies. In cats these tumors are located especially in the small bowel; lymphoma is the most recurring type, followed by carcinoma, leiomyosarcoma and mastocytoma.[12]

In dogs, intestinal tumors are mostly located in the large intestine, like in men. Lymphoma is the most common type. A male predisposition has been reported in both dogs and cats it, with a mean age of 9-10 years. Breed predisposed are: Siamese cat (lymphoma and adenocarcinoma) and German shepherd dogs, collies and giant breeds (adenocarcinoma).[12]

Clinical symptoms depend on the location of the tumor and on the bowel's lumen subocclusion/occlusion. If the neoplasm is located within the small bowel clinical syntoms such as vomiting, hematemesis, anorexia and weight loss can occur. If the tumor is located in the large intestine tenesmus and hematochezia can be present. In case of bowel wall perforation or intussusception abdominal effusion and acute abdominal pain could happen. Lymphoma in the bowel is often transmural, while adenocarcinoma could be annular or pedunculated. At the time of diagnosis, often metastasis or carcinomatosis are present.[12] Other bowel tumors are carcinoid, extramedullary plasmacytoma, mastocytoma, leiomyosarcoma and gastrointestinal stromal tumors. Adenomatous polips are located in the duodenum in cats and in the rectum in dogs.[12]

3. Diagnosis of gastrointestinal tumors: clinico-pathological parameters

In human medicine the use of tumour biomarkers is considered standard practice, and provides clinicians with diagnostic and prognostic information. For tumour markers the priority is to identify true positive cases. Low-invasive biomarkers for obtaining diagnostic and prognostic information may have considerable importance in determining the treatment strategy for patients with neoplastic disease. Several blood-based biomarkers, such as carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP), have been identified as low-invasive biomarkers in human oncology.

Identifying biomarkers for canine neoplastic diseases is important with respect to human oncology as well. Human and canine spontaneous tumours share many essential biological and clinical features, such as their genetics, histopathological morphology, and malignant behaviours to invade and metastasise to other organs. Canine spontaneous tumours have an additional unique feature of maintaining the tumour microenvironment, including tumour heterogeneity, intact immune system, abnormal vasculature, and intratumoral hypoxic gradient, whereas conventional

mouse xenograft models lack these features. Given the similarities and unique features, canine spontaneous tumours have the potential to be useful models in biomarker research; and thus, developing biomarkers for canine neoplastic diseases might help toward the discovery of novel biomarkers for human neoplastic diseases.[13]

The measurement of serum enzyme lactate dehydrogenase (LDH) as an initial screening for differentiating patients with different cancers (i.e. not only for lymphoma) and patients with non-oncological diseases has been used in dogs, but has not produced convincing results.[14] On the other hand, serum LDH levels demonstrated a prognostic value in canine lymphoma recurrence[15] and an LDH increase has been correlated with a worse prognosis in feline lymphoma, similar to the results observed in humans.[16] Therefore, assessing serum total LDH in cats as a possible marker for alimentary lymphoma (AL) may play an important role in differentiating between AL and a non-neoplastic gastrointestinal disease such as inflammatory bowel disease (IBD).

In Veterinary medicine many targets in addition to LDH have been investigated as serum biomarkers for canine lymphoma. Tumor products alpha-fetoprotein (AFP) and microRNAs; biochemical enzymes corticosteroid-induced alkaline phosphatase (ALP); cytokine tumor necrosis factor-α (TNF-α); metabolic profiling by gas chromatography; leakage enzymes thymidine kinase 1 (TK1) and high-mobility group B1 proteins (HMGB1); fucosylated serum proteins as well as serum proteins serum amyloid A (SAA), C-reactive protein (CRP), and haptoglobin have been evaluated. Targets were generally selected as tumor-specific products or as participants in important disease-related pathways. Only TK1, CRP, and haptoglobin have resulted in a commercially available biomarker test.[13]

The thymidine kinases are enzymes that convert deoxythymidine to deoxythymidine monophosphate and have a function in DNA synthesis. Rapidly proliferating cells will have higher levels of thymidine kinase. Serum thymidine kinase activity (sTK) is a useful tumour marker in humans, dogs, and cat with utility as a prognostic indicator in lymphoma.[17]

MicroRNAs (miRNAs), a class of short-noncoding RNAs, play indispensable roles in tumorigenesis by controlling the differentiation, proliferation, and cell death of cancer cells. Cancer cells not only show dysregulation of miRNAs but also release these miRNAs into the bloodstream. The levels of circulating miRNAs can

accurately reflect the number of tumour cells, response to treatment, clinical stages, and tumour grades. In addition, the sequences of mature miRNAs are broadly conserved among diverse species. Therefore, circulating miRNAs have a great potential to be common diagnostic and prognostic biomarkers for both human and canine neoplastic diseases.[18]

Increased serum parathyroid hormone-related peptide (PTHrP) concentration is used to diagnose humoral hypercalcemia of malignancy (HHM) in humans and animals. A commercially available assay for human PTHrP has diagnostic utility in the dog and cats.[19]

In human oncology, the importance of the EGFR/HER-2/KRAS (Fig. 6) signalling pathway in gastric and colorectal cancer tissue is well established. They are protein tyrosine kinases located in cell membranes. The deregulation of these has been shown to play an important role in tumour initiation, progression and metastasis.[20,21]

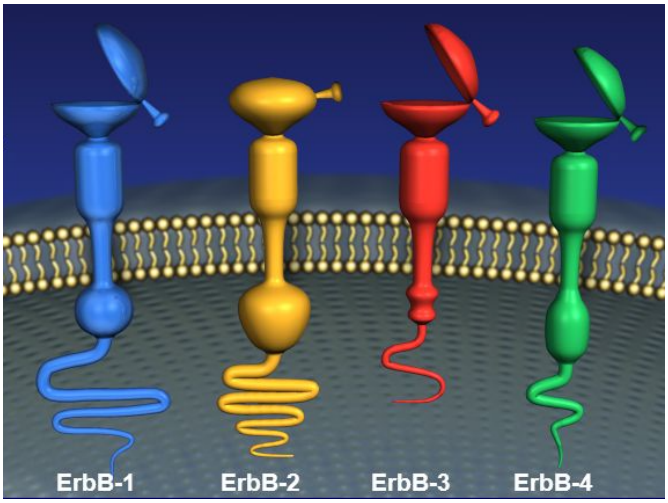

Fig 6. ErbB Receptor Tyrosine Kinases: ErbB-1 (EGFR, HER-1), ErbB-2 (HER-2), ErbB-3 (HER-3), ErbB-4 (HER-4).

The potential relevance of these molecules as prognostic and predictive markers involving the use, in parallel, of in situ hybridization (FISH), and immunohistochemistry (IHC) on tissue samples. Fluorescence in situ hybridization it's a cytogenetic procedure wich use fluorescent probes to detect DNA sequences on chromosome, for example to diagnose many types of chromosomal abnormalities.

Because dogs naturally develop spontaneous cancers, in contrast to mouse models, they constitute an excellent comparative model for cancer. The pathological and behavioural similarities between many spontaneous canine and human tumours make

logical to extend investigations onto molecular oncogenesis in dogs. In veterinary medicine, EGFR protein expression has been detected in numerous tumour types, including canine mammary tumours, primary brain tumours, nasal carcinomas, lung carcinomas and osteosarcomas.[22,23]

We know from literature that these antigens were recognised by the humanized antibodies applied for immunotherapy in human medicine (trastuzumab and cetuximab), leading to growth inhibition in canine mammary cancer cell lines. This finding supports the development of novel targeted therapies and adjuvant strategies for the treatment of EGFR/ HER-2-expressing canine cancers.[24]

The expression of EGFR and HER-2 in canine gastric epithelial tumours was investigated in dogs, to deepen a role of these receptors in carcinogenesis, especially when compared to normal gastric mucosa. Therapeutic targeting of EGFR and HER-2 could be a promising line of research in canine gastric cancer. EGFR protein overexpression in canine and feline intestinal tumors has not been investigated.

4. Diagnosis of gastrointestinal tumors: medical imaging

Medical imaging is an essential part of the diagnostic workup of many gastrointestinal disorders.

Radiography

A native radiographic examination of the stomach includes four projections: ventrodorsal (VD), dorsoventral (DV), left lateral (LL) and right lateral (RL). Air can move in the different part of the stomach and allow to visualize the gastric regions.

On the lateral view, the axis of the stomach from the fundus throughout the body and pylorus is parallel to the ribs. On the VD view of the dog, the cardia, fundus and body of the stomach are located to the left of the midline, and the pyloric portion is located in the right hemiabdomen. The pyloric sphincter is usually located in the right cranial abdominal quadrant around the level of the 10th or 11th rib, usually cranial to the pyloric canal. On the VD view of the cat, the stomach is more acutely angled with the pylorus located at or near the midline. The normal gastric wall appears smooth and uniform and is a few mm thick when fully distended. Rugal folds are more tortuous in the non-distended stomach and become more uniform and parallel to the gastric curvature as distension increases. In the pyloric antrum, they are small and spiral.[25]

The normal small bowel in survey radiography is smooth, continuosly curving tubes or as solid circles or rings. These shapes are produced by contractile activity of the smooth muscle. Segmental contractions give rise to spherical shapes, whereas peristaltic contractions cause long tubular shapes. In the fasted cat gas is rarely present, but in fasted dog 30%-60% of the small bowel content may be gas. The colon of the dog and cat is a thin-walled distensible tube that is divided into ascending, transverse and descending parts. These division are recognised on survey radiographs based on shape, size and location. The shape of the colon is similar to a question mark; the junction between the ascending and tranverse colon is the right colic flexure and the junction between the tranverse and descending colon is the left colic flexure.[26,27]

Contrast radiographic studies should always be preceeded by survey radiographs. Various contrast radiographic techniques have been described for the stomach such as positive-contrast gastrography (CG), using barium or iodinated contrast medium, pneumogastrography (PG), using air or another gas, double-contrast gastrography (DCG), using a positive contrast followed by gas and low-volume gastrography. Small bowel evaluation with contrast medium studies is performed using barium or a low-osmolarity iodinated contrast medium by gastric tube or an oral syringing. For the large intestine, a barium enema, a pneumocolon or a double contrast enema should be used. Contrast studies of the stomach have progressively been replaced by endoscopy while contrast studies of the intestinal tract are still barely performed since the coming of ultrasonography (US) and endoscopy.

Ultrasonography

Transabdominal US is the imaging modality of choice for examination of the abdomen in veterinary medicine. Endoscopic ultrasonography or endosonography (EUS) is useful for studying the stomach and the intestinal wall, but the instruments are very expensive and general anesthesia is required. For US in dogs and cats, the transducers depending on the size of the animals. A microconvex transducer with a frequency range between 5-10 MHz can be used in all animals and may allow visualization of all parts of GI tract. For large and giant-breed dogs, a transducer with a lower frequency (3.5-5 MHz) may be useful to evaluate the deepest part of the stomach. A linear high frequency transducer (7-15 MHz) may be useful to scan the complete stomach and bowel in cats and small dogs and to evaluate the superficial

ventral aspect and left lateral aspect of the stomach and bowel in all animals. The animal should be fasted for 12 hours before the examination so that the stomach is empty or contains gas or fluid but no food. Water administration at 10-15 mL/kg will enhance visibility of mural pathology and may help overcome air-induced artifacts.[28] The abdomen is usually examined in dorsal decubitus. However, for the stomach, because of the presence of gas and fluid, a combination of positions (dorsal, right lateral, left lateral recumbency and standing position) may be required to completely visualize the stomach wall. Right lateral recumbency is useful for pyloric examination and left lateral for the fundus. The echoendoscope (radial and linear multifrequency scanners) is similar to a conventional endoscope except that it has an ultrasound transducer at its tip; EUS allows visualization of both the inner and outer parts of the gastric wall, using high-frequency transducers that can be taken close to the target regions. Transducer coupling is either by direct mucosal contact or inflation of a water-filled balloon surrounding the transducer (Fig. 7).[29] However, up to now, no study has assessed the usefulness of EUS in diagnosing gastric disease in dogs and cats.

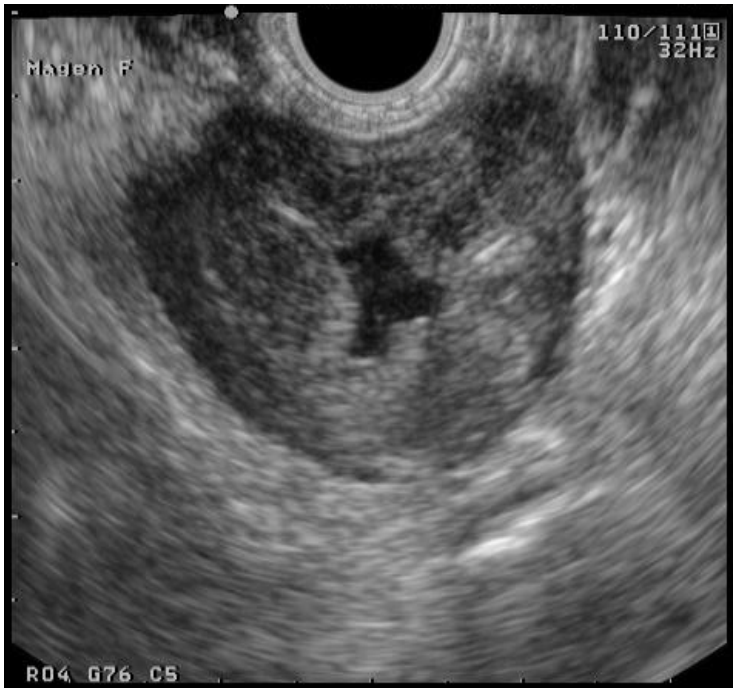

Fig 7. EUS image of the stomach in a dog with gastric adenocarcinoma showing complete loss of stomach wall layering.

On US, different tumor types can show a similar appearance. Loss of layering and a focal thickening are strongly suggestive of a gastric tumor, with a higher sensitivity than radiography.[30] The thickening of the gastric wall can be generalized or localized, symmetrical or asymmetrical, and associated or not with significant loss of motility. A focal thickening with normal layered architecture may suggest leiomyoma, malignant histiocytosis or a polyp. The gastric carcinoma shows a typically asymmetrical, moderate to severe, heterogeneous gastric wall thickening. The normal layers are obliterated but may be replaced by three alternating bands referred to as pseudolayering. Pseudolayering is visible as a poorly defined, echogenic lining on the innermost and/or outermost portions of the gastric wall, separated by a more echoic central zone. It most likely correlates with the unevenly layered tumor distribution noted histopatologically.[31] Myogenic tumors (leiomyoma and leiomyosarcoma) are often located in the pyloric region and appear as hypoechoic submucosal masses arising from the muscular layer. Leiomyoma usually appear as a homogeneous, small, sessile, echoic mass in dogs.[32] A large, complex mass with an irregular central cavity (representing hemorrhage, necrosis or cystic degeneration) most likely represents a leiomyosarcoma.[30] It can ulcerate and lead to wall perforation.[32] Canine and feline gastric lymphoma usually show circumferential, transmural thickening associated with diffuse loss of normal wall layering and reduced wall echogenicity, localized decreased motility and moderate regional lymphadenopathy.[28,33] Lymphoma can also show a focal hypoechoic eccentric mass.[30] The gastric carcinoma is often associated with regional lymphadenopathy. In gastric carcinoma, lymph nodes have a target appearance, with a poorly defined echoic rim and a highly defined echoic center.[31]

The US features of malignant histiocytosis, malignant fibrous histiocytoma and histiocytic sarcoma have been reported sporadically.[34,35] Other tumors, like carcinoid, neurilennoma, nerve-sheath tumor, mast cell tumor and hemangiosarcoma, tend to appear as poorly defined echoic masses or as a focal thickening with loss of layering. There are no specific US features for these tumors.[36]

Benign gastric polyps (hyperplastic and adenomatous) are sessile or pedunculated nodules that arise from the mucosa and protrude into the gastric lumen. In dogs and cats, they may occur as solitary or multiple cauliflower lesions. Occasionally, large polyps in the pyloric antrum can cause gastric outflow obstruction. On US, canine gastric polyps appear as heterogeneous lesions confined to the mucosa without involvement of the submucosal layer. Gastric wall thickening corresponding to wall

edema and luminal fluid accumulation associated with reduced gastric motility may be observed in animals with large lesions. Gastric polyps can rarely display poorly visualized gastric wall layers.[37]

Stomach ulcers are a possible complication of gastric tumors. The US findings associated with gastric ulceration are focal wall thickening with indistinct wall layers, or loss of layering and disruption of the normal mucosal surface in a large ulcer with an ulcer crater. It can be a focal accumulation of hyperechoic material on the mucosal surface (gas bubbles or blood clots) and decreased motility of the affected wall segment (Fig. 8A-E).[30] US-guided fine needle aspiration of the GI tract has been described.[38]

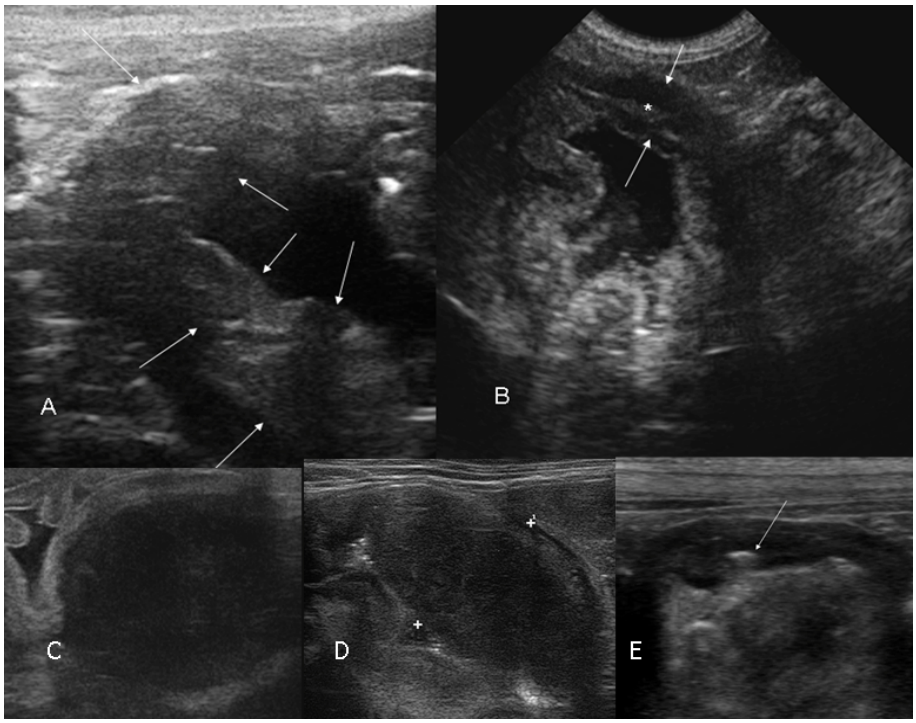

Fig 8. (A) US of the gastric antrum of a dog with adenocarcinoma. There is irregular heterogeneous gastric wall thickening (among arrows) with loss of layering. (B) US of a dog with an adenocarcinoma of the lesser curvature and antrum. In the ventral portion of the stomach wall there is a pseudolayering pattern, with hypoechogenic innermost and outermost parts (arrows), separated by a more echoic central zone (asterisk). (C) Longitudinal US view of dog with leiomyosarcoma of the gastric antrum. A homogenous, round, hypoechoic mass lesion involving the muscular layer of the stomach wall is visible. The mucosa and submucosa are normal. (D) US longitudinal image of the gastric fundus and body of a cat with lymphoma of the stomach. There is a severe circumferential thickening and complete loss of layering of the stomach wall, which is mostly hypoechoic. The wall between the callipers measures approximately 2.5 cm. (E) US image at the level of the gastric body of a dog. Gastric ulceration is visible as a focal wall thickening with loss of layering and disruption of the normal mucosal surface. The ulcer crater is filled with gas (arrow).

As in the stomach, intestinal wall thickening is commonly found with inflammatory diseases or neoplasia. Nonmalignant diseases tends to induce diffuse, mild to moderate wall thickening with preservation of the wall layers. Neoplastic lesion characteristically produce more extensile focal thickening or mass lesions with distruption or loss of the wall layers. The exception is lymphoma, wich can either diffusely infiltrate the bowel or produce mass lesion. In either case there is loss or alteration of the wall layers. Wall thickening may be circumferential. The lesion usually appears distinctly hypoechoic, ulceration may be present, and regional lymphoadenopathy is common. Adenocarcinoma tends to cause circumferential thickening of the bowel with loss of wall layering and partial or complete intestinal obstruction; usually bowel carcinomas are solitary masses but can spread throught the peritoneal cavity.[39] Leiomyomas in the bowel are small and have smooth contours, instead leiomyosarcomas are generally large, eccentric masses. Mast cell disease can produce diffuse wall thickening or a focal mass in cats and dogs. The sonographic characteristics of these abnormalities have not been widely reported.[39] (Fig .9).

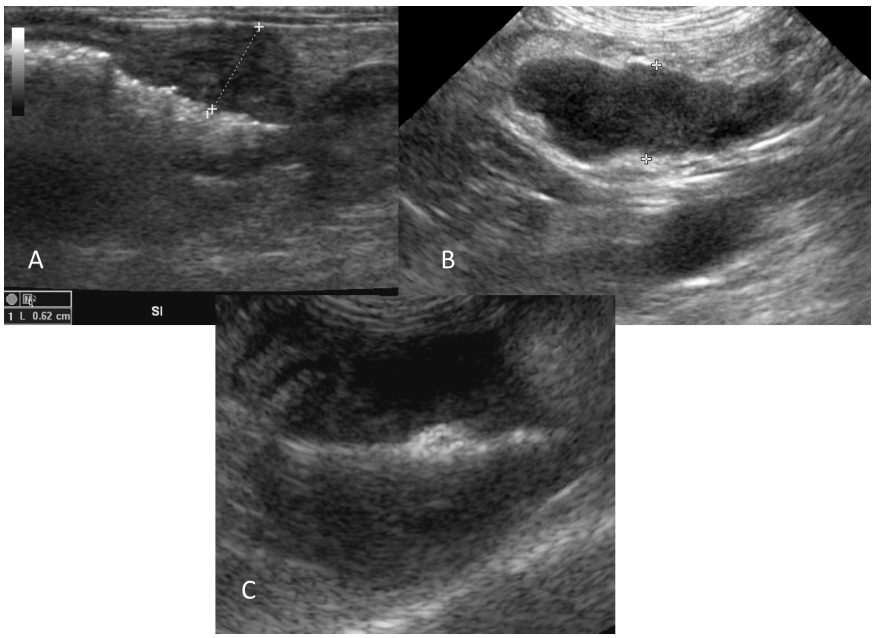

Fig 9. (A) US of a dog with intestinal leiomyosarcoma. There is irregular slightly heterogeneous intestinal wall thickening (among cursors) with loss of layering. (B) Increased in size, hyoechogenic and irregularly outlined metastatic jejunal lymph node. (C) US transversal image of a dog with lymphoma of the intestine (jejunum). There is a severe circumferential thickening and complete loss of layering of the intestinal wall, which is mostly hypoechoic.

Endoscopy

Upper gastrointestinal endoscopy (esophago-gastro-duodenoscopy) is a minimally invasive and traumatic procedure that allows direct visualization of the gastric and intestinal wall and is associated with low morbidity and mortality.[40] A flexible endoscope with an insertion tube with 1 m of working length and a tip with a diameter between 7-9 mm (< 9.5 mm) is recommended. The accessory/biopsy channel should have a minimum diameter of 2.2 mm and the tip should be capable of four-way deflection. The tip must be able to retroflex more than 180° in one plane with a small radius of curvature. The endoscope must have an inflation system (essential for stomach visualization) and a washing lens system. A xenon light source is preferred for greater illumination. Suction is necessary to remove secretions and air; biopsy forceps should be available for tissue collection. The patient should be fasted for at least 12 hours. Water is allowed. If a barium contrast radiographic study has been performed, upper endoscopy should be delayed for 24 hours because barium can cause serious blockage of the instrument.[41] General anesthesia with intubation is required for endoscopy. The patient is positioned in left lateral recumbency because this position facilitates the evaluation of the pylorus. Lubrication of the endoscope is preferred before it is introduced. A mouth gag must be inserted.[41] Gastroduodenoscopy is contraindicated when GI perforation is suspected, the patient is inadequately prepared, the patient cannot be anesthetized or the patient has coagulation disorders. Complications (overdistension of the stomach, perforation due to poor technique, mass rupture and transmission of enteropathogenic organisms) are rare.[41] Gastric polyps are sessile or pedunculated protuberances of the gastric mucosa that do not disappear with maximal inflation. They can be benign (adenomatous polyp), malignant (carcinoma) or inflammatory/hyperplastic. Polyps are most commonly found in the pyloric region, more often in the antrum. At gastroscopy, polyps are generally single, multilobated or grape-like masses that may bleed from an eroded surface and may or may not occlude the pyloric orifice.[42] Endoscopic surgery can be performed to remove polypoid lesions.[43] Gastric neoplasms can present with a variety of appearances, from raised plaques or masses projecting from the lumen to firm, diffusely infiltrating masses invading the stomach wall. They may ulcerate and alter the gastric lumen and shape. Ulcers occur more frequently with adenocarcinoma and leiomyoma/leiomyosarcoma than with lymphoma. Typically, gastric ulcers have raised and thickened margins. The bed of the ulcer blood can be present in the gastric

fundus and may indicate the ulcer's presence. Ulcers resulting from gastric adenocarcinoma are usually associated with broad areas of induration, while ulcers due to leiomyoma are usually crater-like with raised margins. Gastric adenocarcinoma is most commonly seen on the lesser curvature of the stomach. It may appear as an obvious mucosal mass or with mucosal change of color (from pink to purple) with deep pigmentation of the mucosa, ulceration, loss of normal landmarks and rigidity of the gastric mucosa.[44] Sometimes an AC shows marked proliferative changes with a rigid, very dense wall that has completely lost the normal rugal fold architecture. Infiltrative neoplasms, such as lymphoma, usually show a diffusely thickened mucosa with a markedly increased wall consistency (Fig. 10 A-F). However, they can also appear as a mass lesion. Gastric lymphoma can affect either the entire stomach or only part of it, and show a mucosa that is often lumpy and friable or, less commonly, thick and irregular.[41] The indurated mucosa often feels heavy and yields little as the endoscope or biopsy forceps contacts it.[40] If the mass is in the pylorus, it may occlude the pyloric orifice. Leiomyoma usually appear as expansile submucosal masses.[42] All gastric lesions should be biopsied with endoscopic forceps or cytology brushes and multiple samples should be taken for cytology or histopathology.

Endoscopy is useful only if the tumor is located in the duodenum, terminal portion of the ileum, cecum, colon and rectum. The intestinal epithelial neoplasms generally seems polypoid or in mass, with irregular tissue or projecting into the lumen. They may also have ulcerated or plaque appearance. Lymphoma has an invasive attitude and is widespread. Other neoplastic conditions such as leiomyoma, leiomyosarcoma, stromal tumors, mast cell tumor and plasmacytoma have not typical endoscopic criteria.[45]

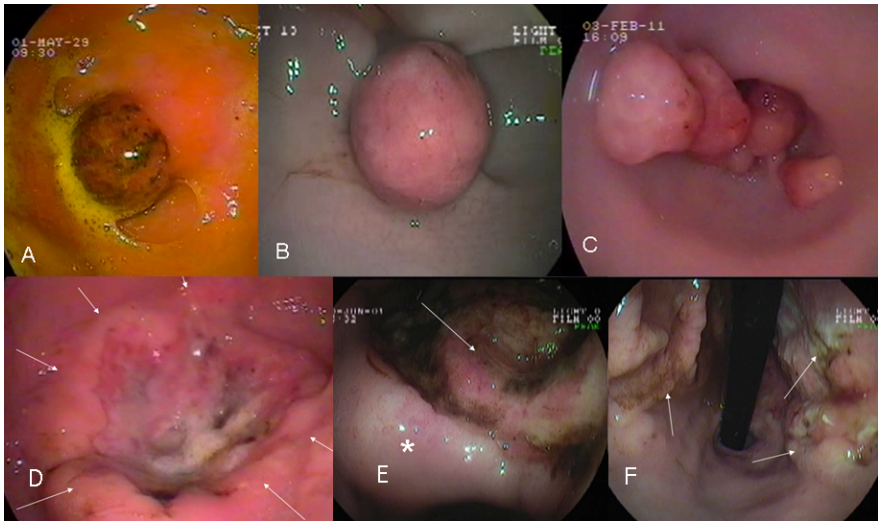

Fig 10. (A) Adenomatous polyp in a dog is visible at the gastric antrum. (B) Large gastric polyp of the antrum (gastric carcinoma in situ) in a dog with chronic vomiting. (C) Gastric antrum grape-like polyps (adenocarcinoma) in a cat with chronic vomiting. (D) Gastric ulcer (adenocarcinoma) in a dog with anorexia and weight loss. Arrows indicate the neoplastic ulcer's borders that surround the necrotic centre. The tumor is located in the gastric body. (E) Gastric carcinoma in a dog. The small curvature (asterisk) is extremely thickened and the fundus folds (arrow) are replaced by proliferative ulcerated neoplastic tissue. (F) Gastric fundus lymphoma in a cat. Arrows indicate fundus rugal folds infiltrated by neoplastic tissue.

Advanced imaging techniques

Computed Tomography (CT), Positron emission tomography (PET) and Magnetic Resonance Imaging (MRI) are used for diagnosis and staging of gastric tumors in human, however so far they are no studies in the veterinary literature. The accuracy of CT in the diagnosis and staging of gastric cancer in humans improves when helical CT of the stomach is performed after the oral administration of water. This procedure, which is termed helical hydro-computed tomography (HHCT), is followed by the intravenous injection of contrast medium to enhance the image of the gastric wall.[46]

Using computed tomography with water as a negative oral contrast agent and intravenous contrast medium, can provide critical information for the diagnosis of gastric diseases. In addition, CT can evaluate the involvement of the gastric wall and extragastric extent of the disease, as compared with gastroenteroscopy and double-contrast upper gastrointestinal study. Regarding lesion location and size, enhancing and growth patterns, presence of calcification or fat, and involvement of the gastric wall and adjacent structures, CT may provide useful information.[47]

The water (500 mL) is administered in a routine procedure to obtain gastric distention before the patient is laid down on the CT table. The dynamic CT imaging for the

gastric lesions is performed in three phases (non-enhanced, arterial, and portovenous). Non-enhanced imaging is obtained to provide a baseline for the degree of lesion enhancement as well as detecting the hemorrhage, calcification, and fat component of the lesion. The arterial phase is obtained 30 seconds after the injection of a dose of 2 mg/kg of nonionic contrast material at a rate of 2.5 mL/s, using an automated power injector. The portovenous phase is obtained 50 s after the contrast injection. The contrast-enhanced phases help in assessing the extent of involvement of the stomach, differentiating mucosal and submucosal tumors, determining the enhancing and growth patterns, and detecting distant metastasis and lymphadenopathy. [47]

The bowel distension is obtained with different CT techniques: CT enteroclysis (CTEc), CT enterography (CTE) and CT colonography (CTC). Fasting and intestine cleansing are necessary for all the procedures. Negative oral contrast agents like lactulose, water, oil emulsion, methylcellulose, polyethylene glycol, mannitol, and ultra–low-dose barium with sorbitol are preferred in the assessment of intestinal wall enhancement. CT enteroclysis is performed after nasojejunal intubation under fluoroscopy; as this process is invasive for patients, CTE is the preferred technique. For this 300 mL 20% w/v mannitol is mixed with 1.5 L water. A total of 500 mL of mixture is given orally to patients in the first 15 minutes followed by 500 mL in the next 15 minutes, then 500 mL in the next 15 minutes. The scan is performed at 50 minutes with the remaining solution given on the table along with intravenous contrast injection to patient.

For CT colonography an oral administration of either water-soluble iodinated contrast medium or a diluted barium sulfate suspension is performed, which is done to tag residual bowel content and differentiate from true colonic lesions. Then colon is inflated with air or carbon dioxide by using a thin and flexible rectal catheter. Intravenous administration of 100 mL of nonionic contrast is performed.[48]

Positron emission tomography with fludeoxyglucose F 18 (^{18}FDG-PET/CT) has rapidly obtained a foothold in the evaluation of bowel disorders, chiefly in neoplasms and inflammatory bowel diseases (IBD). ^{18}FDG-PET imaging relies on the increased uptake and metabolism of ^{18}FDG in inflammation, infection, or neoplasm. Combining ^{18}FDG-PET with CT, both morphologic and functional information regarding disease site and activity can be obtained. This imaging technique has been enhanced further by combining CTE/CTEc and CTC with ^{18}FDG-PET imaging for the evaluation of the small and large bowel, respectively. ^{18}FDG is administered. For PET-CTE,

patients drink 1.5 L of mannitol solution over 45 minutes to 50 minutes, whereas for PET-CTEc, a nasojejunal tube is inserted up to the proximal jejunum and 1.5 L of normal saline is given through it until patients complain of abdominal distension/discomfort. The ^{18}FDG-PET/CTE scanning begins 45 minutes to 60 minutes after injection of ^{18}FDG.

Two techniques of colonic distension have been described when CTC is combined with ^{18}FDGPET imaging: the patients drink 2.5 to 4.0 L of water mixed with polyethylene glycol. Patients are asked to drink this slowly over a period of 1 hour, and scanning is done 45 minutes to 60 minute after intravenous injection of ^{18}FDG. This technique provides adequate colonic distension without the invasiveness of rectal injection. Other technique obtained colonic distension by perrectal injection of 2 to 3 L of tap water.[48]

Magnetic resonance imaging–related procedures include routine MR imaging abdomen, MR enteroclysis, and MR enterography. MR imaging is now increasingly being used for the evaluation of bowel pathology in view of the lack of ionizing radiation and superior contrast resolution. The potential disadvantages include longer acquisition times, higher cost, and motion artifacts. Motion artifacts can be eliminated partially with the use of fast sequences and antispasmodic agents, such as hyoscine, which decreases bowel movement. Although MR enteroclysis provides better mucosal detail, it involves nasojejunal intubation, which is an invasive procedure and involves ionizing radiation during fluoroscopy-guided tube insertion. MR enterography (MRE) is currently the preferred technique as it is faster and more tolerable. Positive contrast agents are gadolinium-chelates, ferrous/manganese ions, milk, blueberry juice, green tea, and ice cream. Negative contrast agents are: ferumoxsil oral suspension, oral supermagnetic particles, and perfluorooctyl-bromide. Biphasic contrast agents (water-based): water, diatrizoate meglumine, diatrizoate sodium salt, mannitol, locust bean gum, low-dose barium, manganese compounds, and Macadamia 16 (hydrophilic liquid emollient derived from ethoxylated Macadamia oil glycerides).[48]

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CHAPTER II

SCIENTIFIC AIMS

Gastrointestinal (GI) neoplasia is uncommon in dogs and cats, with gastric tumors representing <1% and intestinal tumors <10% of overall neoplasia in these species. GI neoplasms tend to be malignant. Early diagnosis of GI neoplasia is challenging because of the lack of specificity of the clinical signs.

Clinico-pathological parameters and medical imaging are non-invasive ways that provide a diagnosis of GI tumors in dogs and cats. Huge improvements have been made the last decennia in the field of biomarkers and diagnostic imaging providing new perspectives for diagnosis, prognosis and treatment in veterinary oncology. However, to date, only a limited number of studies have made use these opportunities.

Therefore, the aim of this thesis was to provide new insights in the diagnosis of GI tumours in dogs and cats allowing an improved diagnosis, prognostic capabilities and therapeutic planning.

Specific objectives of this thesis were:

1. To evaluate EGFR and HER-2 immunohistochemical expression and KRAS mutational status in dogs with gastric tumours, as a step to improve the treatment of dogs with gastric tumours.

   These epidermal growth factor receptors and analogs correlate with prognosis and response to therapy in many human cancers. More in-depth knowledge of the role of these molecular pathways could provide new insights into the treatment of canine gastric tumours but also into the evaluation of dogs as a comparative model for human cancers.

2. To determine whether an elevation of the total LDH can be used to differentiate alimentary lymphoma (AL) from non-neoplastic gastrointestinal diseases in cats.

   The clinical presentation of feline AL is quite similar to that of inflammatory bowel disease (IBD). It has been suggested that an increase in total LDH can be used as a prognostic factor in cats with lymphoma. Potentially the total LDH can also be used to help

differentiate malignant from non malignant disease presented with similar symptoms.

3. To describe and optimize helical hydro-computed tomography (HHCT) in dogs and cats and to evaluate its applicability in clinical patients.

   Performing a helical CT after oral administration of water (HHCT) should improve the visibility of the gastric wall and allow a more accurate diagnosis of gastric tumours in dogs and cats. However, the procedure has to be first optimized, as the optimal amount of water had to be determined and its feasibility has to be determined. This was demonstrated in normal animals, after with its use in clinical patients was evaluated.

CHAPTER III

EGFR, HER-2 AND KRAS IN CANINE GASTRIC EPITHELIAL TUMORS: COMPARATIVE ONCOLOGY

Adapted from:

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4.

1. Abstract

Epidermal growth factor receptor (EGFR or HER-1) and its analog c-erbB-2 (HER-2) are protein tyrosine kinases correlated with prognosis and response to therapy in a variety of human cancers. KRAS mediates the transduction of signals between EGFR and the nucleus, and mutation in this gene have been associated with resistance to anti-EGFR drugs. In human oncology, the importance of the EGFR/HER-2/KRAS signaling pathway in gastric cancer is well established, and HER-2 testing is required before initiating therapy. Conversely, this pathway has never been investigated in canine gastric tumours. A total of 19 canine gastric epithelial neoplasms (5 adenomas and 14 carcinomas) have retrospectively been evaluated for EGFR/HER-2 immunohistochemical expression and KRAS mutational status. Five (35.7%) carcinomas were classified as intestinal-type and 9 (64.3%) as diffuse-type. EGFR was overexpressed (≥1+) in 8 (42.1%) cases and HER-2 (3+) in 11 (57.9%) cases, regardless of tumour location or biological behaviour. The percentage of EGFR-positive tumours was significantly higher in the intestinal-type (80%) than in the diffuse-type (11.1%, $p = 0.023$). *KRAS* gene was wild type in 18 cases, whereas one mucinous carcinoma harboured a point mutation at codon 12 (G12R) (Also know as NC_006609.03:c.36G>C following HGVS nomenclature). EGFR and HER-2 may be promising prognostic and therapeutic targets in canine gastric epithelial neoplasms. As mutation in the KRAS gene has been found, future studies might investigate the role of KRAS in gastric tumours further. Additional studies are necessary to evaluate the role of dog as a model for human gastric cancer.

2. Introduction

Gastric tumours are rare in dogs, representing less than 1% of all canine malignancies[1,2]; 70–80% are epithelial adenocarcinomas[1]. As in humans, canine gastric carcinoma is more prevalent in males and is usually fatal[2-5]. Gastric adenomas are mainly incidental findings; they may undergo malignant transformation[1].

In humans, gastric and gastro-oesophageal junction (GEJ) adenocarcinomas are one of a major cause of cancer morbidity and mortality worldwide[6].

Complete surgical resection is the mainstay of treatment for non-metastatic diseases, but many patients are not diagnosed until their disease is either locally advanced or metastatic and therefore unresectable[6]. The prognosis in advanced stages is very poor[7].

One of the most significant innovative targets in human cancer is the HER family. The members of this family, EGFR, c-erbB-2, c-erbB-3 and c-erbB-4 (also known as HER-1, HER-2, HER-3 and HER-4, respectively) are normally located in cell membranes and consist of an extracellular ligand-binding domain and an intracellular domain with tyrosine kinase activity[8]. The EGF signaling pathway has been studied in human cancer patients with particular attention paid to EGFR, HER-2 and their activating ligands as the deregulation of these has been shown to play an important role in tumour initiation, progression and metastasis[7,8]. Tumor dependence on specific molecular pathways may identify the best target for therapy exploration. Activation of the epidermal growth factor receptor (EGFR)-related signaling pathways drives numerous cancer-promoting processes, such as cell proliferation, apoptosis inhibition, angiogenesis, cell adhesion, and motility and invasion, and also controls the development of drug resistance. Therefore, anti-EGFR approaches (antibodies directed against the extracellular domain and small inhibitors of the tyrosine kinase activity) have been one of the most successful examples of molecular target therapy in human solid neoplasias.

Aberrant HER-2 expression or function has been implicated in gastric carcinogenesis and in other tumor types, including breast, ovarian, salivary gland, prostate and lung cancers[9]. Trastuzumab, a recombinant humanised IgG1*k* monoclonal antibody directed against the HER-2 extracellular domain, has shown an improvement of survival in patients with metastatic HER-2 overexpressing gastric cancer[7-10], providing significant benefits in terms of response rate, median progression-free survival and overall survival[11]. Currently, trastuzumab in combination with chemotherapy is considered the standard treatment for patients with HER-2-positive advanced GC, and immunohisto chemistry is the primary HER-2 testing method: a score of 3+ confirms eligibility for trastuzumab therapy, whereas a 1+ score indicates no overexpression. The interpretation of tumours scoring 2+ is open to debate, and FISH has been proposed to confirm HER-2 overexpression in such cases[7-12].

EGFR overexpression in primary gastric carcinomas and/or metastases has also been reported and is linked to a poor prognosis[13–15]. An increasing interest has been shown

in developing immunohistochemistry-based screening methods to select patients who are eligible for treatment with cetuximab, an anti-EGFR monoclonal antibody used in advanced colorectal cancer[15,16].

Signal transduction between HER receptors and the nucleus is mediated by a small protein encoded by the KRAS gene[17]. In human oncology, the importance of the EGFR/HER-2/KRAS signalling pathway in GC is well established. KRAS mutations activate RAS proteins that continuously stimulate the signalling pathways in the absence of upstream stimulation of EGFR. Consequently, tumours bearing a KRAS mutation are less likely to respond to anti-EGFR drugs[17].

In veterinary medicine, EGFR protein expression has been detected in numerous tumour types, including canine mammary tumours, primary brain tumours, nasal carcinomas, lung carcinomas and osteosarcomas[18–22]. HER-2 overexpression is reported in 19% to 35% of canine mammary neoplasms [23– 25]. In a recent comparison study, Singer et al. studied canine EGFR and HER-2 expression and biology using mammary cancer cell lines, observing a substantial similarity between human and canine EGFR and HER-2 tumour-associated antigens. The antigens were recognised by trastuzumab and cetuximab antibodies, leading to growth inhibition in canine mammary cancer cell lines. This finding supports the development of novel targeted therapies and adjuvant strategies for the treatment of EGFR/ HER-2-expressing canine cancers[26]. Conversely, the EGFR/ HER-2/KRAS pathway has never been investigated in canine gastric tumours.

The aim of the present study was to evaluate EGFR and HER-2 immunohistochemical expression and KRAS mutational status in a series of canine gastric tumours. More in-depth knowledge of the role of these molecular pathways could provide new insights for the treatment of canine gastric tumours and for the evaluation of dogs as a comparative model for human cancers.

3. Materials and methods

Ethics Statement

This study is a retrospective investigation carried out on archived tissue samples from dogs with gastric tumours. As the research did not influence any therapeutic decision in human subjects, approval by an Ethics Committee was not required. However, all

diagnostic and therapeutic procedures were performed in accordance with the "Public Health Service Policy on Humane Care and Use of Laboratory Animals".
All the examined samples were collected for diagnostic purposes as part of routine standard care. Owners gave informed consent to the use of clinical data and stored biological samples for teaching and research purposes.

Selection Criteria

Formalin-fixed and paraffin-embedded (FFPE) tissue samples from dogs with a histological diagnosis of benign or malignant epithelial gastric tumours were retrieved from the archives of the Department of Veterinary Medical Sciences, University of Bologna, Italy, the Veterinary Oncology Center, Sasso Marconi, Italy, and the Faculty of Veterinary Medicine, Ghent University, Belgium. Only primary benign and malignant epithelial gastric tumours, as confirmed by clinical and/or post-mortem findings, were used. Endoscopic biopsies and surgical or post-mortem samples have also been included. Reference haematoxylin-eosin stained sections were reviewed by an expert veterinary pathologist (GB) to confirm the original diagnosis and to standardise the pathological classification according to WHO guidelines for the classification of gastrointestinal tumours of domestic animals[27]. Tumours were divided into two main histological groups for statistical purposes: intestinal-type (comprising tubular/papillary adenocarcinomas) and diffuse-type (comprising signet ring/mucinous carcinomas and anaplastic carcinomas)[28]. Only tissue sections containing more than 50% of tumour cells were included in the study. If available, tumour stage according to the tumour node metastasis (TNM) classification proposed by the American Joint Committee on Cancer was recorded[29]. EGFR/ HER-2 expression was also investigated in 10 samples of normal canine gastric mucosa (from the archives of the Department of Veterinary Medical Sciences, University of Bologna, Italy).

EGFR and HER-2 Immunohistochemistry

EGFR and HER-2 expression was detected by immunohistochemistry (IHC) using commercial anti-human antibodies whose reactivity in canine tissues has previously been validated[19–30]. Four-micrometer-thick FFPE tissue sections were de-waxed and rehydrated. Endogenous peroxidase activity was blocked by incubation for 30 min with 3% hydrogen peroxide in distilled water. For EGFR, antigen retrieval was

obtained by incubating sections in a 0.05% protease XIV (from Streptomyces griseus, P5147-1G, Sigma Aldrich, MO, USA) solution (pH 7.5) for 15 min at 37°C. Subsequently, sections were incubated for 60 min at 37°C in a humid chamber with the primary antibody (EGFR Ab-10, clone 111.6; mouse monoclonal; NeoMarkers, Fremont, CA, USA) diluted 1:100 in phosphate buffered saline (PBS; pH 7.4, 0.01 M). Sections were incubated with a high sensitivity detection kit (EnVision Plus-HRP, Dako, Glostrup, Denmark), according to the manufacturer's instructions.

For HER-2, antigen was retrieved with citrate buffer (2.1 g citric acid monohydrate/litre distilled water), pH 6.0, and heated for two 5-min periods in a microwave oven at 750 W. Sections were treated with a protein-blocking solution (Protein Block Serum-Free, Dako) for 20 min. Tissue sections were then incubated overnight at 4°C with the primary antibody (anti c- erb-B2 oncoprotein; rabbit polyclonal, Dako) diluted 1:50 in a solution of 1% bovine serum albumin and PBS. Sites of primary antibody binding were identified using a streptavidinbiotin-peroxidase kit (LSAB, Dako) and diaminobenzidine was used as chromogen (0.04% for 10 min at room temperature).

Sections were then counterstained with Papanicolaou's hematoxylin. Sections of canine mammary carcinomas were used as positive controls, as we know they express the tested antigens. Negative controls were obtained by substituting the primary antibody with an unrelated serum.

The immunohistochemical expression of EGFR was graduated as follows: 0, no staining observed or membrane staining in <1% neoplastic cells; 1+, weak complete or incomplete membrane staining in >1% neoplastic cells; 2+, moderate complete or incomplete membrane staining in >1% neoplastic cells; 3+, strong complete or incomplete membrane staining in >1% neoplastic cells. Cases with an EGFR score ≥1+ were considered positive[31]. HER-2 was evaluated according to the criteria proposed by Hofmann et al[32] for human GC. HER-2 positivity (3+ IHC reaction) was defined as strong, complete or basolateral membranous immunoreactivity in at least 10% of tumour cells. In biopsies, a cluster (approximately five or more) of positively stained cells was considered positive[12]. EGFR and HER-2 expression was further evaluated in biopsy samples of gastric mucosa from ten dogs without gastric tumor.

DNA Extraction and KRAS Gene Analysis

FFPE block-derived sections from gastric lesions were reviewed for quality and cellular content. DNA was extracted from 5-µmFFPE sections. Cells were lysed in 50 mM KCl, 10 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$ and Tween-20 0.45%, with the addition of Proteinase K at a concentration of 1.25 mg/ml, overnight at 56°C. Proteinase K was inactivated at 95°C for 10 minutes after which samples were centrifuged twice to eliminate debris. Supernatant was assessed for DNA quality and quantity by Nanodrop (Celbio, Milan, Italy) and was then submitted to PCR amplification.

Exon 1 of canine *KRAS* gene was amplified by PCR using the following primers: forward 5'-CTGCACTGAATTTTCTGAAGCA-3' and reverse 5'-AAAATGGGCCTGCACAAAT'-3'. PCR products were purified using the Minielute PCR purification kit (Qiagen, Hilden, Germany) and then submitted to sequencing using the BigDye Terminator 3.1 Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequence reactions were purified using the DyeEx 2.0 Spin kit (Qiagen) and separated by capillary electrophoresis with laser-induced fluorescence detection (3130 Genetic Analyzer, Applied Biosystems). Sequencing products were analysed in comparison with the wild type sequence of the gene.

Statistical Analysis

The association between EGFR/HER-2 expression (positive/negative) and clinical pathological parameters (tumour stage, location, malignancy and histotype) were tested for significance with Fisher's exact test. Significance was set at $p<0.05$. Tests were carried out with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

4. Results

Nineteen samples have been collected (7 endoscopic biopsies, 8 surgical samples and 4 post-mortem samples). There were five benign tumours (26.3%; 1 tubular adenoma, 1 papillary adenoma, and 3 tubulopapillary adenomas) and 14 gastric carcinomas (73.7%; 1 in situ carcinoma, 2 tubular adenocarcinomas, 1 papillary adenocarcinoma, 1 tubulopapillary adenocarcinoma, 8 mucinous/signet ring cell carcinomas and 1 undifferentiated carcinoma). There were 5 intestinal-type (35.7%) and 9 diffuse type

carcinomas (64.3%) according to Lauren's classification[28]. Tumours were located in the cardia (n = 1; 5.9%), gastric fundus (n = 2; 10.5%), lesser curvature (n = 6; 31.6%) and pyloric antrum (n = 8; 42.1%). In two (10.5%) cases, tumour location was unavailable. No statistical association has been found between tumour location and histological type. Grossly, diffuse-type tumours most often appeared as a diffuse thickening of gastric mucosa (linitis plastica), while intestinal-type tumours frequently had the appearance of polypoid-like lesions or mucosal plaques with ulceration. Among malignant tumours, 3 were stage 0, 4 were stage I, 2 were stage III and 4 were stage IV. In one case the staging was unavailable. The mean age of the affected dogs was 10.8 years for adenomas and 8.8 for carcinomas (range 3–16 years); 12 dogs were males (63.2%) and 7 were females (36.8%). There were 3 Boxers, 2 Shih-tzus, 5 crossbreeds, and 9 dogs of other breeds. Animal details are summarised in Table 1.

Table 1. Animal details and tumor characteristics in 19 cases of canine gastric epithelial neoplasms.

Case	Breed	Age, yrs	Sex	Tumor location	Type of sample	Histological diagnosis	TNM Stage*	EGFR score	HER-2 score	KRAS gene analysis
1	Shih-tzu	8	M	Pyloric antrum	Surgical	Tubular adenoma	N/A	0	2+	Wild type
2	Shih-tzu	10	MC	Pyloric antrum	Surgical	Papillary adenoma	N/A	3+	3+	Wild type
3	Crossbreed	7	M	Pyloric antrum	Surgical	Tubulopapillary adenoma	N/A	0	3+	Wild type
4	Crossbreed	13	M	Pyloric antrum	Surgical	Tubulopapillary adenoma	N/A	2+	3+	Wild type
5	Crossbreed	16	FS	Cardia	Endoscopic biopsy	Tubulopapillary adenoma	N/A	1+	2+	Wild type
6	Boxer	3	M	Pyloric antrum	Surgical	In situ carcinoma within tubulopapillary adenoma	TisN0M0/Stage 0	1+	3+	Wild type
7	Italian Hound	8	F	Pyloric antrum	Post mortem	Tubular adenocarcinoma	T3NxM1/Stage IV	1+	3+	Wild type
8	Chow-Chow	12	M	Lesser curvature	Post mortem	Tubular adenocarcinoma	T3N1M0/Stage IIIA	3+	3+	Wild type
9	Crossbreed	7	FS	–	Endoscopic biopsy	Papillary adenocarcinoma	T1NxM0	0	2+	Wild type
10	Italian Griffon	12	F	–	Post mortem	Tubulopapillary adenocarcinoma	T2NxM1/Stage IV	1+	3+	Wild type
11	Boxer	9	M	Gastric fundus	Post mortem	Mucinous/signet-ring cell carcinoma	T3N1M0/Stage IIIA	0	2+	Wild type
12	Boxer	10	M	Gastric fundus	Surgical	Mucinous/signet-ring cell carcinoma	T4N1MX/Stage IV	0	3+	Mut G12R
13	Pyrenean Mountain Dog	10	F	Lesser curvature	Endoscopic biopsy	Mucinous/signet-ring cell carcinoma	T1N0M0/Stage IA	0	3+	Wild type
14	German Shepherd	10	F	Pyloric antrum	Endoscopic biopsy	Mucinous/signet-ring cell carcinoma	T1N1M0/Stage IB	0	3+	Wild type
15	Dalmatian	10	F	Lesser curvature	Surgical	Mucinous/signet-ring cell carcinoma	T4N1M0/Stage IV	1+	3+	Wild type
16	Golden Retriever	8	M	Lesser curvature	Surgical	Mucinous/signet-ring cell carcinoma	T2N0M0/Stage IB	0	2+	Wild type
17	Shar Pei	6	M	Pyloric antrum	Endoscopic biopsy	Mucinous/signet-ring cell carcinoma	T1N1M0/Stage IB	0	2+	Wild type
18	Bouvier des Flandres	9	M	Lesser curvature	Endoscopic biopsy	Mucinous/signet-ring cell carcinoma	T1NxM0	0	2+	Wild type
19	Crossbreed	10	M	Lesser curvature	Endoscopic biopsy	Undifferentiated carcinoma	N/A	0	–	Wild type

N/A, not applicable;
*TNM staging system for gastric carcinomas according to the classification of the American Joint Commission on Cancer[11].
doi:10.1371/journal.pone.0085388.t001

Table 1. Breed, age, sex, tumor location, type of sample, histological diagnosis, tumour node metastasis (TNM) stage, EGFR and HER-2 and KRAS gene analysis of the animals.

EGFR and HER-2 Expression

The EGFR protein was expressed in 8 (42.1%) out of 19 samples. Five (26.3%) cases were scored as 1+, 1 (5.3%) case was scored as 2+ and 2 (15.8%) cases were scored as 3+ (Figure 1a,b). No differences were observed in EGFR expression on the basis of tumour stage, location or malignancy. The percentage of EGFR-positive lesions was significantly higher among intestinal-type (80%) than diffuse-type (11.1%, p = 0.023) tumors (Table 2). Eleven (57.9%) tumours were considered HER-2 positive (3+). In the remaining cases (n = 7; 36.9%), HER-2 expression was classified as 2+ (Figure 1c,d). In one dog (case 19), HER-2 analysis was not possible because of tissue exhaustion. No statistically significant differences in HER-2 positivity were observed on the basis of clinical pathological parameters (Table 2). In adenomas, HER-2 staining was more intense in dysplastic areas.

Parameter	EGFR ≥1+ (n= 8)	P	HER-2 3+ (n= 11)	P
Sex		ns		ns
Male	4/12		6/11	
F*emale*	4/7		5/7	
Age, years		ns		ns
< 10 years	2/9		3/9	
> 10 years	6/10		8/9	
Localisation		ns		ns
Pyloric antrum	4/8		6/11	
Malignancy		ns		ns
Adenomas	3/5		3/5	
Carcinomas	5/14		8/13	
Histotype		0.023		ns
Intestinal type	4/5		4/5	
Diffuse type	1/9		4/8	
Stage IV		ns		ns
Yes	3/4		4/4	
No	2/9		3/9	

Table 2. Association between EGFR/HER-2 status and clinic-pathological parameters in 19 canine gastric epithelial neoplasms.

EGFR/HER-2 expression was not detected in the 10 samples of normal gastric mucosa, with the exception of scattered foci of weak basolateral positivity (Figure 1e,f).

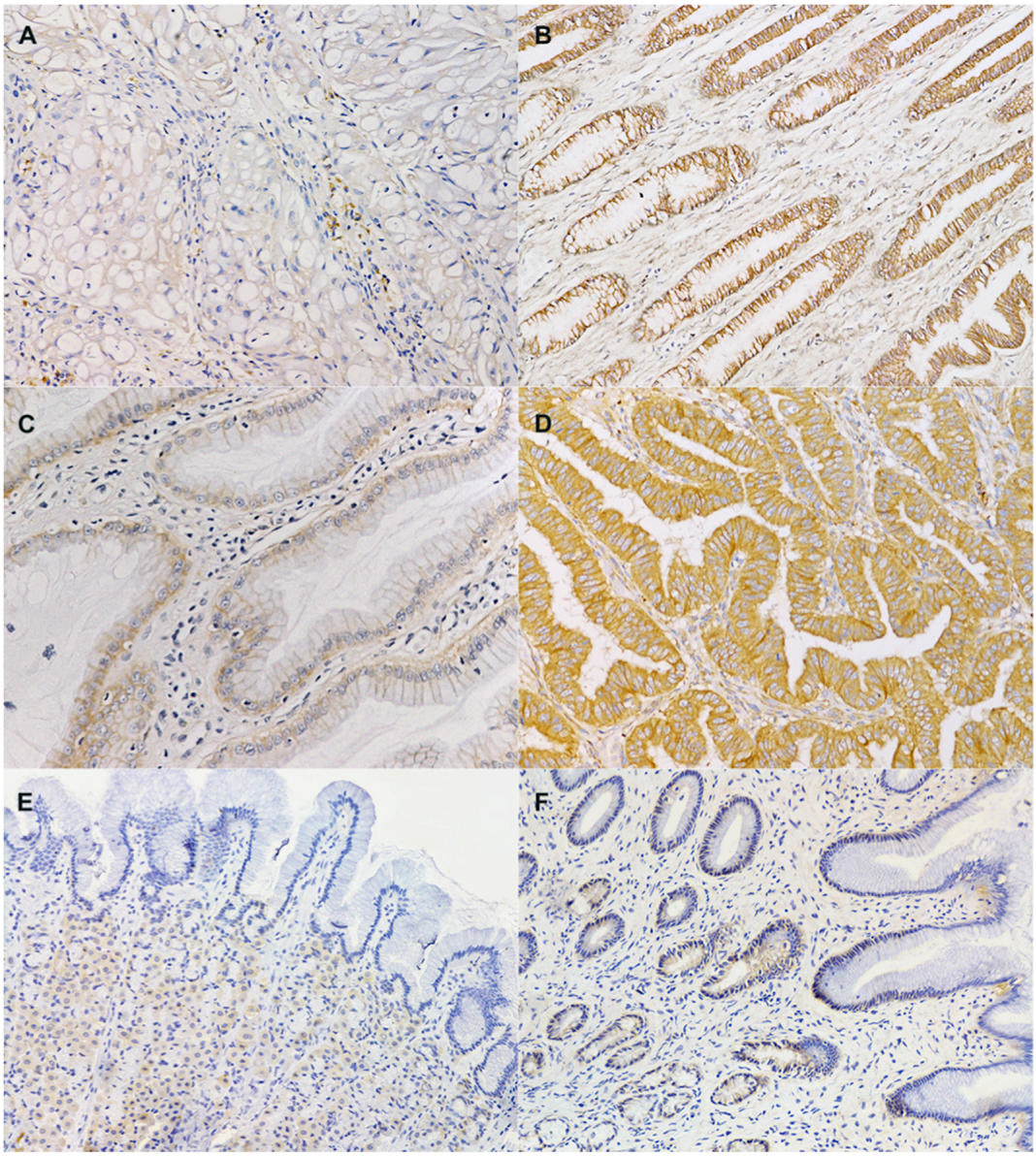

Fig 1. EGFR and HER-2 immunohistochemistry of canine samples. (A). Dog, stomach. Signet ring cell carcinoma (case No. 15). EGFR immunohistochemistry. Faint and partial membrane labelling of the neoplastic cells (1+). Haematoxylin counterstain. 200x. (B) Dog, stomach. Papillary adenoma (case No. 2). EGFR immunohistochemistry. Strong and complete membrane labeling of the neoplastic cells (3+). Haematoxylin counterstain. 100x. (C) Dog, stomach. Tubulopapillary adenoma (case No. 5). HER-2 immunohistochemistry. Moderate basolateral membrane labeling of the neoplastic cells (2+). Haematoxylin counterstain. 200x. (D) Dog, stomach. In situ tubulopapillary carcinoma (case No. 6). HER-2 immunohistochemistry. Strong and complete membrane labeling of the neoplastic cells (3+). Haematoxylin counterstain. 200x. (E) Dog, stomach, fundus. EGFR immunohistocheistry. Negative labeling of the mucosal epithelium. Faint granular cytoplasmic positivity of the parietal cells. Haematoxylin counterstain. 200x. (F) Dog, stomach, pyloric antrum. HER-2 immunohistochemistry. Scattered foci of faint basolateral positivity. Haematoxylin counterstain. 200x.

KRAS Gene Analysis

All five adenomas showed a wild type *KRAS* gene status. Of the 14 carcinomas, 13 were *KRAS* wild type and one was mutated. The identified mutation was a G to C transversion at the first position of codon 12 of the gene (GGT ⓟCGT (G12R)) (Also know as NC_006609.03:c.36G>C following HGVS nomenclature[33]) which induces the substitution of a glycine with an arginine. The mutation was found in stage IV mucinous/signet-ring cell carcinoma (Figure 2).

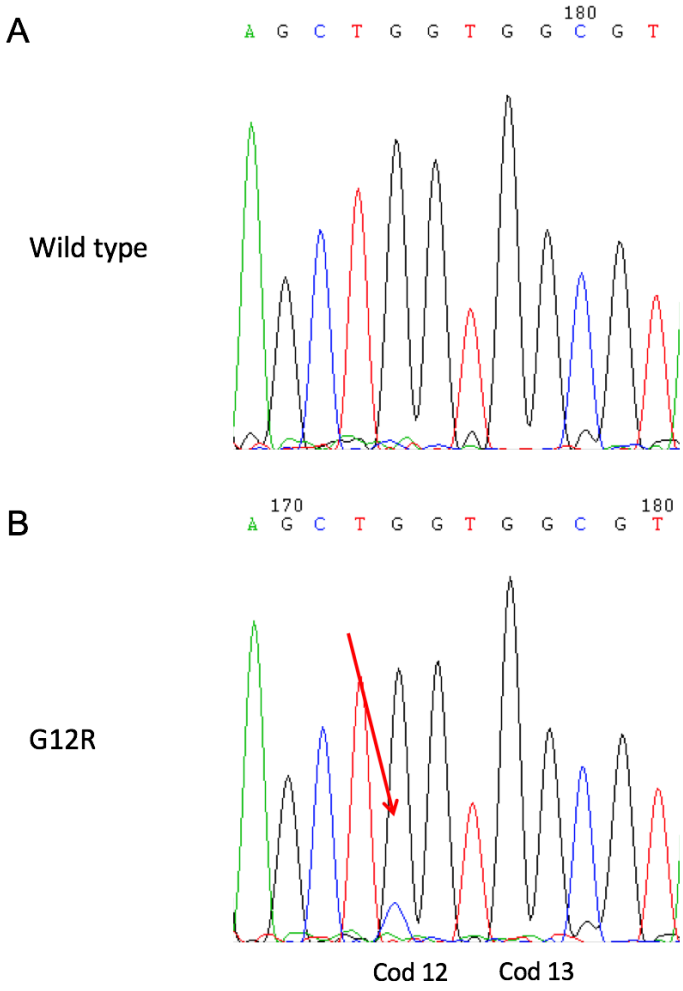

Fig 2. KRAS analysis. Mutation analysis performed by direct sequencing on wild type (A) and mutated (B) samples. Arrows indicate the point mutation.

5. Discussion

HER-2 and EGFR are transmembrane tyrosine kinases that can promote tumour genesis and progression. Expression of HER-2 and EGFR appear to be closely

related, and one or both proteins are frequently overexpressed in gastric epithelial cancer cells[8]. In recent years, new developments in cancer biology have led to the emergence of novel molecular-targeted therapeutics. These drugs act selectively on cancer cells at a molecular, biochemical and genetic level, specifically targeting abnormal cells, with minimal effects on the function of normal cells[8]. As in humans, the prognosis of canine gastric cancer is very poor and currently available therapeutic aids fail to significantly prolong survival. The need for new treatment options thus encourages in-depth studies on the role of these molecules as potential therapeutic targets in veterinary medicine as well. Additionally, strong similarities have been observed between human and canine gastric tumour with regard to clinical presentation and histopathological features, which indicates the dog as a potential comparative model for human GC.

Our retrospective study was conducted on 19 cases of canine gastric epithelial tumours, the small number of cases possibly constituting a limitation. Data on clinical stage were not available in six dogs (nos. 1, 2, 3, 4, 5 and 19). A 1.7:1 male to female ratio was observed, confirming the male predisposition previously reported by other authors[2–5]. Additionally, our data support the preferential localisation of these tumours to the pyloric antrum and lesser curvature[34–36].

Overall, both receptors were expressed in a high percentage of cases (EGFR: 42%, HER-2: 61%). All HER-2 negative cases were also negative for EGFR expression exept one (case 5), whereas a subset of EGFR negative tumours was found among HER-2 positive tumours. Immunohistochemical expression was membranous, complete or basolateral. EGFR positivity among tumors was extremely heterogeneous, therefore we did not observe major intra-tumour differences in HER-2 expression.

In humans, HER-2 immunohistochemical expression varies according to tumour location, with a higher rate of HER-2 positivity in GEJ tumours compared to those located in the gastric body (34% vs 20%)[11]. The single most important factor for the development of these tumours is the mucosal irritation caused by chronic GE reflux. In such case, squamous epithelium is eroded and replaced by columnar epithelium, either by intestinal metaplasia or by the extension of columnar epithelium from the stomach (Barrett's oesophagus)[36,37].

HER-2 expression in humans also differs significantly on the basis of histological subtype. Intestinal-type cancers usually exhibit higher rates of HER-2 positivity

compared to diffuse-type tumors (34% vs 6%)[9]. No clear correlation has been found between EGFR expression and tumour location or histotype.

Among the cases in this study, only one tumour was located in the GEJ (HER-2, 2+); the remaining lesions were almost equally distributed between the gastric fundus and the pyloric antrum, with no significant difference in receptor expression. Conversely, a higher percentage of intestinal-type compared to diffuse-type carcinomas were positive for both markers. However, this difference was only statistically significant for EGFR.

In human GC, HER-2 is involved in the development of relatively early-stage carcinogenesis[12]. Additionally, although the majority of studies conducted in human medicine links the overexpression of HER-2 to adverse prognosis[12], a small number of these studies has not found any correlation with the biological behaviour of the tumour[38]. Likewise, EGFR expression has been associated with increased tumour aggressiveness[15]. Given the retrospective nature of this study, it was not possible to trace the clinical follow-up of the majority of cases. Nevertheless, some indication about tumour biological behaviour can be inferred from IHC expression in gastric mucosa samples from controls compared to those from adenomas and carcinomas.

Neither receptor was expressed in non-neoplastic gastric mucosa, showing their possible involvement in carcinogenesis. Additionally, in benign tumours, HER-2 was more intensely expressed in the focal areas of dysplasia, suggesting its potential relevance in premalignant lesions. However, as no appreciable differences in marker expression has been observed between adenomas and carcinomas and between locally advanced and metastatic cancer, a correlation with tumour biological behaviour would seem unlikely.

In human oncology, it has been observed that the *KRAS* gene is affected by a limited number of mutations, more than 90% involving codons 12, 13 and 61 (less relevant). These mutations are associated with the constitutive activation of the gene and are considered responsible for resistance to treatment with anti-EGFR monoclonal antibodies[39].

In gastric carcinomas, the reported frequency of *KRAS* point mutations is between 8% and 10%[40,41]. Although *KRAS* mutations represent a prognostic factor for colorectal and lung cancer, their correlation with the biological behaviour of gastric tumours is still poorly defined[39–41].

The nucleotide sequence of the canine *KRAS* gene is close to identical to the human one, and the resulting amino acidic sequence is identical[42]. Kraegel and coworkers analysed *KRAS* activation in a subset of canine non-small cell lung cancers: despite the wide disparity in the incidence of non-small cell lung cancer between dogs and humans, the frequency of *KRAS* point mutation was similar. Although species-specific factors may be responsible for mutations, exposure to common environmental carcinogens may account for some of the identified similarities in *KRAS* activation[42].

Mutation analysis of *KRAS* performed on our samples showed the presence of a single mutation at codon 12. This mutation is among the most frequently detected in humans and results in the substitution of a glycine (the only amino acid without a side-chain) by an amino acid with a side-chain (arginine), thus leading to a geometric alteration of the protein. This results in a lack of GTP hydrolysis which keeps *KRAS* in a permanently activated state[43]. The mutation was found in a mucinous carcinoma of the gastric fundus characterised by a massive infiltration of adjacent structures and regional lymph node metastases. Unfortunately, no information was available on the clinical course of the case, a 10- year-old male boxer. The potential presence of *KRAS* mutation in dogs should be taken into account when considering to use of targeted drugs against the HER pathway as it could represent a mechanism of resistance[44].

In conclusion, the present study revealed a high expression of EGFR and HER-2 in canine gastric epithelial tumours, which suggests a role of these receptors in carcinogenesis, especially when compared to the constant negativity of normal gastric mucosa. Additionally, there was a significantly higher percentage of EGFR positive cases among intestinal-type carcinomas. Unlike humans, however, we did not observe a relationship between marker expression and anatomical location or the biological behaviour of tumours. Finally, a codon 12 mutation in the *KRAS* gene was identified, equivalent to those found in human gastric carcinomas, suggesting that this altered pathway may also exert a role in the pathogenesis of gastric cancer in dogs.

The potential relevance of these molecules as prognostic and predictive markers indicates the need for further studies on larger case series involving the use, in parallel, of in situ hybridisation and immunohistochemistry.

In conclusion, the pathological and behavioural similarities between many spontaneous canine and human tumours make logical to extend investigations into molecular oncogenesis to dogs. Therapeutic targeting of EGFR and HER-2 could be a

promising line of research in canine gastric tumour. Further studies are needed to evaluate the role of the dog as a model for human GC.

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CHAPTER IV

IS SERUM TOTAL LDH EVALUATION ABLE TO DIFFERENTIATE BETWEEN ALIMENTARY LYMPHOMA AND INFLAMMATORY BOWEL DISEASE IN A REAL WORLD CLINICAL SETTING?

Adapted from:

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1. Abstract

An increase in enzyme lactate dehydrogenase (LDH) in serum is a negative prognostic factor for survival in cats affected by lymphoma. Measuring LDH at the time of diagnosis has been studied for differentiating neoplastic disease from non-neoplastic disease in dogs. Inflammatory bowel disease (IBD) and alimentary lymphoma (AL) are common diseases in cats.

The aim of this study was to determine whether elevation of total LDH occurred in cats with AL and non-neoplastic gastrointestinal (GI) disease, such as IBD, and to evaluate whether this enzyme is useful in supporting the differential diagnosis of these specific diseases.

A retrospective controlled multicenter study was carried-out in a real world setting of three Italian private veterinary clinics. Fifty-seven client-owned cats with a history of chronic GI symptoms were enrolled; 25 cats were histologically diagnosed as having AL and 32 cats as having IBD. Serum samples of total LDH analysis have been measured.

Serum total LDH were significantly higher (P=0.017) in cats with AL (median: 300 U/L; IQR: 172-351 U/L) than in cats with IBD (median: 191 U/L; IQR: 135-239 U/L). In particular, serum total LDH values were significantly different between cats with AL and those with IBD in neutred males (P = 0.001) while in younger cats (up to 7 years) the difference was close to the significant level (P = 0.088). A significant diagnostic accuracy was found in the overall population (68.6%; P=0.017) with a best cut-off value of serum total LDH ranging from 239 to 243 U/L. As far as the accuracy in subgroups of cats is concerned, serum total LDH showed good capability of differentiating these two conditions in neutered males (P=0.002) (AUC: 0.889; sensitivity: 88.9%; specificity: 84.6%; PPV: 80.0%; NPV: 91.7%).

Although our study showed that gender and age are important factors in differentiating serum total LDH between cats with AL and those with IBD, this test presents a fair diagnostic accuracy in differentiating between these two conditions in the overall population.

2. Introduction

Lactate dehydrogenase (LDH) is an enzyme which catalyzes the last step of the conversion of pyruvate to lactate during anaerobic glycolysis. Lactate dehydrogenase is found in the cells of almost all body tissues. There are five isoenzymes with LDH activity. Different normal human tissues contain different patterns of these five isoenzymes[1]. Alteration in LDH isoenzymes levels has been observed during development, under changing biological conditions and in response to pathological processes. Lactate dehydrogenase activity in serum increases as a marker of cellular necrosis[2].

Serum LDH and its isoenzyme distribution have been studied extensively in various body cancers. Malignant tumor tissue or contiguous tissue damaged by a tumor liberates enzymes into circulation which contribute towards an abnormal increase in enzyme levels[3]. Regardless of tissue origin, studies on LDH distribution in different types of malignant tumors have shown LDH to be abnormally increased[3]. Moreover, LDH has also been identified as a valuable predictive and/or prognostic biomarker for different types of carcinoma[4]. Serum LDH is commonly increased in patients with hematopoietic malignancies, such as Hodgkin's lymphoma, non-Hodgkin's lymphoma or multiple myeloma[5]. Lactate dehydrogenase is one of the risk factors included in the International Prognostic Index, and it is considered to be a strong predictor of survival in patients with aggressive lymphoid cancers[6]. The total LDH level in the blood may be a meaningful diagnostic parameter in human cancer patients, but the clinical significance of serum isoenzymatic patterns of LDH is still under discussion; the LDH-5 isoform has been added to the list of highly promising targets for cancer treatment[5].

In veterinary medicine, LDH has been evaluated in dogs, and it has been demonstrated that LDH levels have limited use in differentiating dogs with cancer from both healthy dogs and dogs with non-neoplastic diseases, as well as in differentiating among different types of tumors[7]. On the other hand, the determination of LDH activity may help in identifying episodes of recurrence in dogs with lymphoma[8]. In feline oncology, it has been reported that an increase in serum LDH activity in the initial chemistry profile was a negative prognostic indicator for survival in cats with lymphoma[9]. As far as isoenzymes are concerned, it has been reported that

LDH-5 has a dominant concentration and LDH-1 is very scant in normal feline leukocytes, thus suggesting that the leukocytes of cats tend to produce more energy under anaerobic conditions compared to the leukocytes of dogs or rabbits[10].

Lymphomas are a common malignancy affecting cats[11], representing 30% of feline tumors[12]. Alimentary lymphoma is the most common form of lymphoma in cats[13]. A majority of the T-cell immunophenotype (83%) in feline GI lymphoma suggests that previous studies may have underestimated lymphoma incidence, due to the difficulty in distinguishing mucosal T-cell lymphoma from lymphoplasmacytic IBD[12]. The clinical presentation of feline AL is quite similar to feline IBD[14], a chronic enteropathy characterized by persistent or recurrent GI symptoms and histologically confirmed inflammation[15].

The aims of this study were: 1) to determine whether elevation of total LDH occurred in cats with AL and non-neoplastic GI diseases, such as IBD, and 2) to evaluate whether this enzyme is useful in supporting the differential diagnosis of these specific diseases.

3. Materials and methods

Experimental Design

All consecutive client-owned cats with a history of chronic GI symptoms, such as weight loss, anorexia, vomiting, and small bowel diarrhea, referred to three Italian private veterinary clinics (“Giardini Margherita” Veterinary Clinic, Bologna; “Modena Sud” Veterinary Clinic, Spilamberto, Modena; ”Argentina” Polyclinic, Arma di Taggia, Imperia) were selected for a prospective controlled multicenter study carried out between January 2013 and September 2015.

The serum LDH levels were compared between AL and IBD cats, and the diagnostic accuracy in differentiating between these two diseases was evaluated. The possible effect of gender and age was also taken into account.

All owners gave their oral informed consent for the enrollment of their cats into the study. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals (1996)" prepared by the National Academy of Sciences. Ethical approval was not required since no animal research was involved in the study and the data were collected from the records of cats managed

according to the usual clinical practice in a real world setting of three veterinary centers.

Selection Criteria

All cats underwent a physical examination, fecal flotation assays, fecal enzyme-linked immunosorbent assay (ELISA) immunoassay for Giardia, a complete blood count, a biochemistry profile, urinalysis, FIV-FeLV serologic ELISA test and serum total T_4. Abdominal ultrasonography was performed in all patients to exclude non-gastrointestinal causes for their clinical symptoms and to study the GI features.

Exclusion Criteria

Cats which responded to treatment for Giardiasis (fenbendazole 50 mg/kg per os once a day for 5 days) or to specific diets (hydrolyzed protein diet, etc.) were excluded in order to exclude adverse food reaction. Patients with primary renal, hepatic or pancreatic disease, hyperthyroidism, diabetes, toxic causes or other intestinal parasitic diseases as well as patients which palpable abdominal lesion or lymphadenopathy appreciable on palpation were also excluded from the study.

Diagnostic Procedures

All the cats underwent abdominal ultrasonographic (US) examination and endoscopic GI biopsy to collect stomach and duodenum histological samples in order to differentiate between the AL and the IBD groups. Endoscopies were performed by two endoscopists (R.T. and E.B.). Ileoscopy and ileum biopsy were not routinely performed in the centers involved in the study. A histopathological diagnosis of all bioptic samples was carried out according to the histopathological standards of GI inflammation of endoscopic bioptic samples published by the World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization group[16,17]. The IBD cases were classified as mild, moderate and severe[18]. Cases of lymphomas were graded applying the modified World Health Organization (WHO) classification for lymphoma[19,20] according to the following method. Lymphomas with 0 to 5 mitoses/400x field were classified as low grade, those with 6 to 10 mitoses/400x field were classified as medium grade and those with greater than 10 mitoses/400x field were classified as high grade[14]. Immunohistochemistry was required if it was needed to confirm the presence of GI lymphoma.

Immunohistochemistry

Two sections 4 µm-thick were cut from the paraffin blocks and labeled by immunohistochemistry with antibodies anti-CD3 (clone CD3-12, Leukocyte Antigen Biology Laboratory, UC Davis School of Veterinary Medicine, Davis, CA, USA) and anti-CD79 (clone HM57, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The sections were dewaxed in toluene and rehydrated. Endogenous peroxidase was blocked by immersion in H_2O_2 0.3% in methanol for 30 min. The sections were then rinsed in Tris buffer, and the antigen was retrieved with citrate buffer (pH 6.0, CD3 sections) and with ethylenedinitrilotetraacetic acid (EDTA) buffer (pH 8.0, CD79 sections) by heating for two 5-min periods in a microwave at 750 W, followed by cooling at room temperature for 20 min. The antibodies CD3 and CD79 were diluted in phosphate buffered saline (PBS) 1:20 and 1:750, respectively. All antibodies were incubated with the tissue sections overnight at 4°C. Binding sites were revealed using a secondary biotinylated antibody (dilution 1:200) and amplified using a commercial avidin-biotinperoxidase kit (VECTASTAIN ABC Kits, Vector Laboratories, Ltd., Peterborough, UK). Chromogen DAB (3,30diaminobenzidine; 0.05% for 3 minutes at room temperature) was used. The slides were counterstained with Papanicolaou hematoxylin. The primary antibody was replaced with an irrelevant, isotype-matched antibody as a negative control. Reactive lymph nodes were used to assess the specificity of the immunohistochemical procedure.

LDH Evaluation

The serum total LDH was evaluated at the time of clinical presentation after 12 hours of fasting in all cats. Blood was taken from the jugular vein and the serum was obtained by centrifugation after 20 minutes.

A photometric method for assessing total LDH was carried out in one laboratory (Olympus analyzer; IDEXX Laboratories S.r.l., Milan, Italy; reference range: 0–182 U/L; intra- and inter- assay coefficients of variation (CVs): unavailable) while a kinetic optimized method (SCE, LDH-P; BT1500 VET; Futurlab S.r.l., Limena, PD, Italy; reference range: 63–273 U/L; intra- and inter-assay CVs: 1.69–3.86% and 1.26–2.13%, respecively) was used in the other two laboratories.

The values detected by means of the photometric method were proportionally adjusted to the upper reference limit of the kinetic optimized method (273 U/L).

Statistics

Median, interquartile range (IQR), range and frequencies were reported as descriptive statistics. Age and weight were described as scale variables and were also dichotomized for analysis: a cut-off value of 7 years was chosen for age[21,22,23] while weight was dichotomized according to the median value (4 kg).

The Fisher's exact, the Pearson's chi-square and the linear-by-linear chi-square tests were applied to analyze the dichotomous, nominal and ordinal discrete variables, respectively, while the Kruskal-Wallis test, the Wilcoxon/Mann-Whitney rank test and the Spearman rank correlation were used for analyzing scalar variables.

The diagnostic accuracy of serum total LDH values in differentiating AL from IBD cats was evaluated by means of the area (AUC) under the receiver operating characteristics (ROC) curve. The standard error (SE) of the AUC was computed by means of a distribution- free non-parametric method while the best cut-off value of serum total LDH was identified by means of a maximum likelihood ratio (LR) method according to the following formula: LR = (Frequency of true positive + Frequency of true negative) / (Frequency of false positive + Frequency of false negative)[24]. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were evaluated using the best cut-off value.

Data were managed and analyzed by means of the IBM SPSS Statistics package (version 23.0; IBM Co., Armonk, NY, USA) and a two-tailed P value equal to 0.05 was chosen as the limit of statistical significance.

4. Results

Patients

Fifty-seven client-owned cats were used: 52 European shorthair cats, 1 Birman cat, 2 Maine coons, 1 Norwegian forest cat and 1 Persian. Twenty-two (38.6%) were neutered males and 35 (61.4%) were spayed females; median age was 10 (IQR: 7.5-12) years, range: 1–16 years (n = 43, 75.4% were over 7 years of age) and the median weight was 4.0 (IQR: 3.0-4.5), range: 2–10 kg (n = 31, 54.4% weighed more than 4 kg). Abnormalities detected on physical examination included poor body condition (body condition score, BSC: 1-2/5) (n = 25; 43.9%), poor coat condition (n = 26; 45.6%), lethargy (n = 11; 19.3%), diffusely thickened intestinal loops (n = 16;

28.1%), and gaseous or liquid intestinal matter (n = 16; 28.1%). Fecal flotation assays and fecal ELISA immunoassays for the detection of Giardia were negative in all cases.

Clinicopathological abnormalities included mild anemia (n = 17; 29.8%), mild neutrophilia (n = 15; 26.3%) and mild hypoalbuminemia (n = 6; 10.5%) as well as mild elevations of azotemia (n = 9; 15.8%), bilirubin (n = 8; 14.0%) and liver enzymes (n = 5; 8.8%). All the cats had a negative serologic ELISA test for retrovirus except for one which was FeLV positive (1.8%). Serological T4 was normal in all cats.

Study Groups

Twenty-five cats had a diagnosis of AL (43.9%) and 32 cats a diagnosis of IBD (56.1%). Of the 25 AL cats, 16 were low grade (64.0%) and 9 were high grade (36.0%) (no cats with intermediate grade were diagnosed). The lymphoma was located in the stomach in 17 out of 25 cats (68.0%), in the small bowel in 7 cats (28.0%) and in both organs in 1 cats (4.0%). Immunophenotyping on histological samples was available in 24 AL cats (96.0%); T-lymphoma was present in 15 of these cats (62.5%) and B-lymphoma was present in 9 cats (37.5%).

Of the 32 IBD cats, 6 (18.8%) had mild disease, 14 had moderate disease (43.8%) and 12 cats had severe disease (37.5%).

Ultrasonographic examination revealed moderate to severe GI wall thickening together with loss of layering in the 9 cats with high grade lymphoma (100%), mild increased wall thickening (muscular layer) with normal layering in 22 cases (11 cases in the 16 cats with low grade lymphoma, 68.7%; 11 cases in cats with IBD, 34.4%), and normal wall thickening and normal layering in 26 cats (5 cases in the 16 cats with low grade lymphoma, 31.3%; 21cases in cats with IBD, 65.6%). Mild to moderate mesenteric lymph node enlargement was also visible in 25 cats, 18 with lymphoma (72.0%) and 7 with IBD (21.9%) (Table 1).

The upper GI endoscopy of the 9 cats with high-grade lymphoma showed an abnormal appearance of the gastric mucosa in 8 cases (88.9%); there was decreased distensibility of the gastric body also after insufflation, mild to moderate hyperemia, increased granularity and friability) and focal or linear erosion or ulcers in 4 cases (44.4%). No relevant alterations of the gastric mucosa were found in low-grade

lymphoma and IBD cats. In the small bowel of all the cats, the mucosa was friable and irregular, with increased graininess and coerced villi (Table 1).

Table 1. Ultrasonographic (US) and endoscopic (EDS) findings.

	Total	**AL Low grade**	**AL High grade**	**IBD**
	57	**16**	**9**	**32**
US Wall thickening				
- Mod/Sev	9	0	9 (100%)	0
- Mild	22	11 (68.7%)	0	11 (34.4%)
- Normal	26	5 (31.3%)	0	21 (65.6%)
US Node enlargement	25	18/25 (72.0%)		7 (21.9%)
EDS GI abnomality	8	0	8 (88.9%)	0
EDS GI erosions	4	0	4 (44.4%)	0

The characteristics of the two study groups are shown in Table 2. Breed, gender and body weight were not significantly different between the two groups while the AL cats were older than the IBD cats: in fact, the majority of them were over 7 years of age (n = 23/25, 92.0% *vs*. n = 20/32, 62.5%; P = 0.013).

Table 2. Characteristics of the two study groups.

	All cases (n=57)	**Alimentary lymphoma (n=25)**	**IBD (n=32)**	***P value***
Severity of the disease:	-	16 (64.0%) Low grade 9 (36.0%) High grade	6 (18.8%) Mild 14 (43.8%) Moderate 12 (37.5%) Severe	-
Breed:				*P=0.061* [a]
- European shorthair	52 (91.2%)	25 (100%)	27 (84.4%)	
- Others	5 (8.8%)	0	5 (15.6%)	
Gender:				*P=0.788* [a]
- Neutered males	22 (38.6%)	9 (36.0%)	13 (40.6%)	
- Spayed females	35 (61.4%)	16 (64.0%)	19 (59.4%)	
Age (years):				
- Median (IQR)	10.0 (7.5-12.0)	10.0 (9.0-12.0)	9.0 (5.5-11.8)	*P=0.118* [b]
- >7 years	43 (75.4%)	23 (92.0%)	20 (62.5%)	*P=0.013* [a]
Weight (kg):				
- Median (IQR)	4.0 (3.0-4.5)	4.0 (3.3-4.5)	4.0 (3.0-4.4)	*P=0.758* [b]
- 4 kg or more	31 (54.4%)	13 (52.0%)	18 (56.3%)	*P=0.794* [a]

[a] Fisher's exact test

[b] Wilcoxon/Mann-Whitney rank test for independent samples

Distribution of LDH in AL and IBD Cats

The distribution of the serum total LDH values observed in AL and IBD cats is shown in Fig 1; the descriptive statistics, stratified according to the characteristics of AL and IBD cats, are reported in Table 3. Median standardized serum total LDH values were higher in cats with lymphoma than in those with IBD: 300 (QR: 172-351) in AL *vs*. 191 (IQR:135-239) in IBD. The comparison between AL and IBD cats was statistically significant (P = 0.017), as well as a significantly (P = 0.002) higher frequency of cats with serum total LDH values higher than the upper reference limit was found in AL cats (n = 16, 64.0%) than in IBD cats (n = 7, 21.9%).

Fig 1. Distribution of serum total LDH values in alimentary lymphoma and IBD cats. Data are reported as U/L adjusted to the upper reference limit of the kinetic optimized method method (273 U/L).

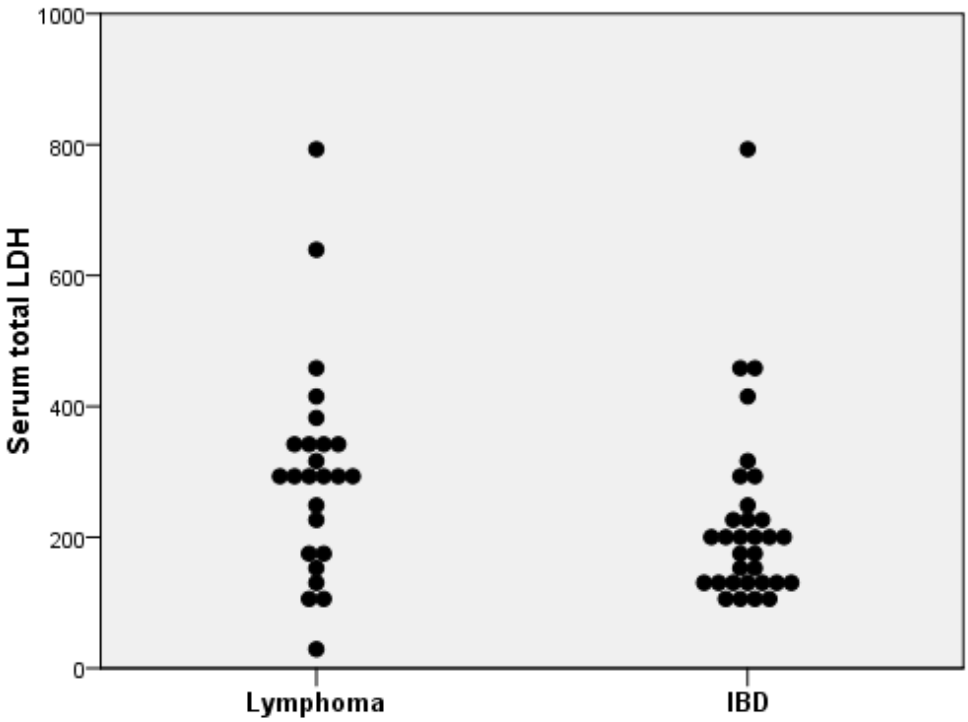

Table 3. Descriptive statistics of serum total LDH values (U/L) according to the characteristics of alimentary lymphoma and IBD.

	Descriptive statistics		Abnormal values
	Median (IQR)	**Range**	
Alimentary lymphoma (n=25)	**300 (172-351)**	**29-800**	**16 (64.0%)**
Location:			
- Stomach (n=17)	302 (164-351)	29-465	11 (64.7%)
- Small bowel (n=7)	291 (168-640)	114-800	4 (57.1%)
- Stomach and small bowel (n=1)	203 (-)	-	1 (100%)
	P=0.990 [a]		*P=0.702* [b]
Immunophenotype:			
- T-lymphoma (n=15)	291 (131-302)	29-800	8 (53.3%)
- B-lymphoma (n=9)	374 (296-368)	152-465	8 (88.9%)
	P=0.096 [c]		*P=0.178* [d]
Grade:			
- Low grade (n=16)	267 (140-301)	29-800	8 (50.0%)
- High grade (n=9)	347 (296-368)	152-465	8 (88.9%)
	P=0.074 [c]		*P=0.088* [d]
IBD (n=32):	**191 (135-239)**	**95-786**	**7 (21.9%)**
Grade:			
- Mild (n=6)	158 (116-246)	103-289	1 (16.7%)
- Moderate (n=14)	179 (130-238)	95-786	2 (14.3%)
- Severe (n=12)	203 (140-387)	98-454	4 (33.3%)
	P=0.309 [f]		*P=0.328* [e]

[a] Kruskal-Wallis test
[b] Pearson chi-square
[c] Wilcoxon/Mann-Whitney rank test for independent samples
[d] Fisher's exact test
[e] Liner-by-linear chi-square
[f] Spearman rank correlation

Although no significant relationships were found between serum total LDH values and the characteristics of both AL and IBD cats (Table 3), higher median total LDH values were found in cats with high grade AL (347 U/L, IQR: 296-368 U/L *vs*. 267 U/L, IQR: 140-301 U/L in low grade; P = 0.074) and in those with B-lymphoma (374 U/L, IQR: 296-368 U/L *vs*. 291 U/L, IQR: 131-302 U/Lin T-lymphoma; P = 0.096) as well as a non significant higher frequency of abnormal LDH values was found in cats with severe IBD (33.3% *vs*. 16.7% and 14.3% in mild and moderate IBD, respectively; P = 0.328).

Table 4 shows the detailed results of the analyses carried out within the various classes of gender and age. In AL cats, the spayed females (P = 0.037) had significantly lower serum total LDH values than neutered males, while no significant

differences were found for gender in IBD cats (P = 0.472). On the other hand, serum total LDH values were significantly higher in older than in younger IBD cats (P = 0.048). In addition, significantly higher values of LDH in AL versus IBD cats were found in neutered males (P = 0.001) while in cats up to 7 years of age the difference was only close to the significant level (P = 0.088).

Table 4. Comparison of serum total LDH values (U/L) between alimentary lymphoma and IBD cats. Data are shown as median values and interquartile ranges (IQRs).

Tested factors	**Alimentary lymphoma (n=25)**	**IBD (n=32)**	**P value**
Gender (All cases):			
- Neutered males	353 (289-525)	156 (125-224)	0.001
- Spayed females	267 (136-326)	191 (139-285)	0.523
P value	*0.037*	*0.472*	-
Age (All cases):			
- Up to 7 years	351 (350-ND)	137 (118-207)	0.088
- Over 7 years	293 (168-347)	203 (159-274)	0.165
P value	*0.240*	*0.048*	-

ND: not detectable

Diagnostic Accuracy

The ROC analysis showed a significant overall diagnostic accuracy (AUC±SE = 0.686±0.075; P = 0.017; Fig 2). The maximum LR value (2.71) identified the best cut-off as ranging from 239 to 243 U/L with a sensitivity, specificity, PPV and NPV: 68.0% (17/25), 78.1% (25/32), 70.8% (17/24) and 75.8% (25/33), respectively.

Fig 2. Receiver operating characteristics (ROC) curve of serum total LDH values in differentiating AL from IBD cats. The best cut-off (corresponding to a serum total LDH value ranging 239-243 U/L with a LR value equal to 2.71) is identified in the ROC curve.

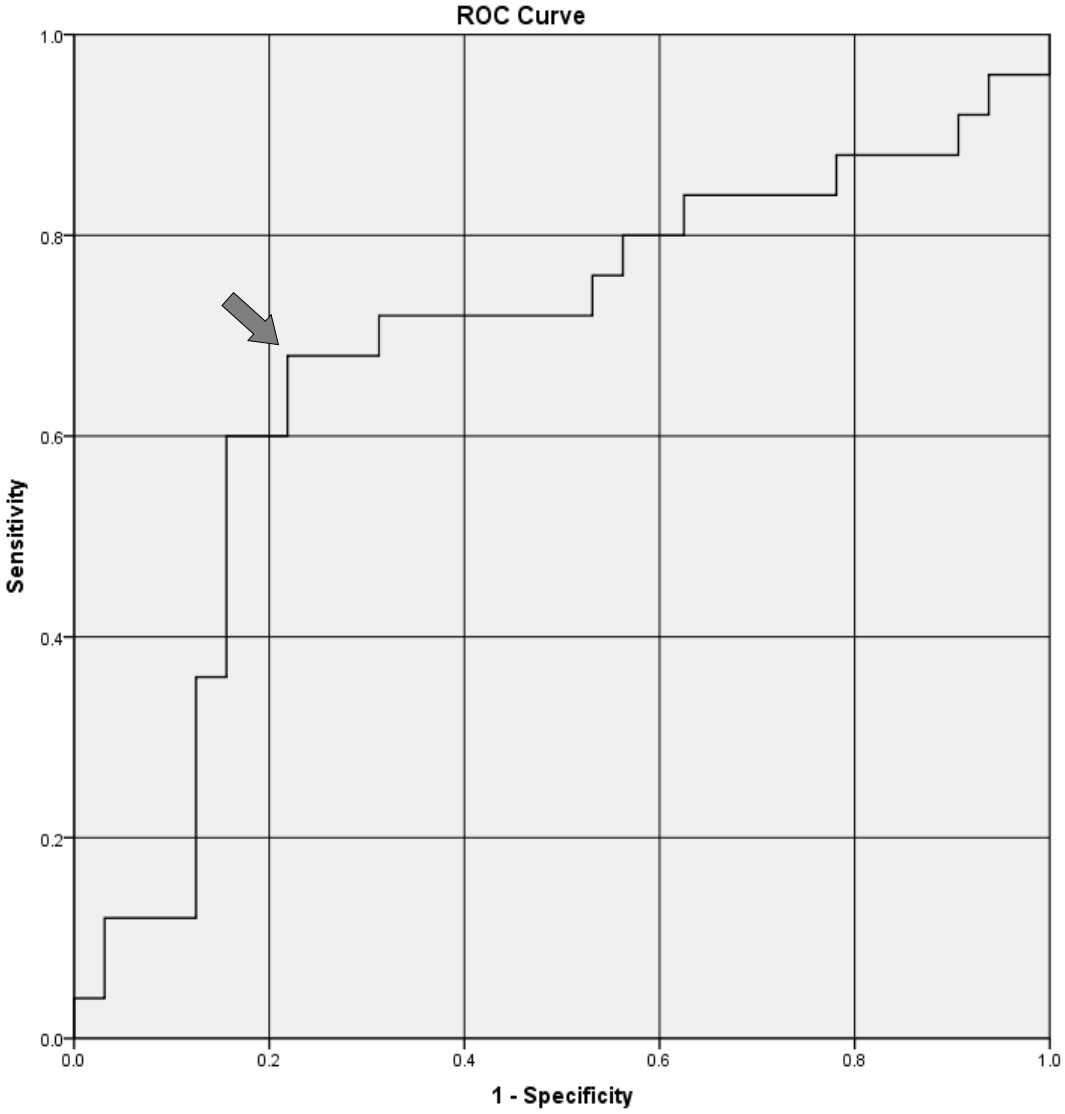

Fig 3 and Table 5 show the results of the ROC analysis stratified according to the characteristics of the cats studied. Gender (P = 0.010) and age (P = 0.014) significantly conditioned the diagnostic accuracy; in fact, significant values of accuracy were only found in neutered males (88.9%; P = 0.002) while in cats up to 7 years of age it was only close to the significant level (91.7%; P = 0.068). In particular, sensitivity prevailed both in neutered males (88.9%) and in younger cats (100%); these figures provided good values of NPV in both groups (91.7% in neutered males and 100% in cats up to 7 years of age).

Fig 3. Receiver operating characteristics (ROC) curves of serum total LDH values in differentiating AL from IBD cats stratified according to gender (a) and age (b). The best cut-off values are also identified in the ROC curves.

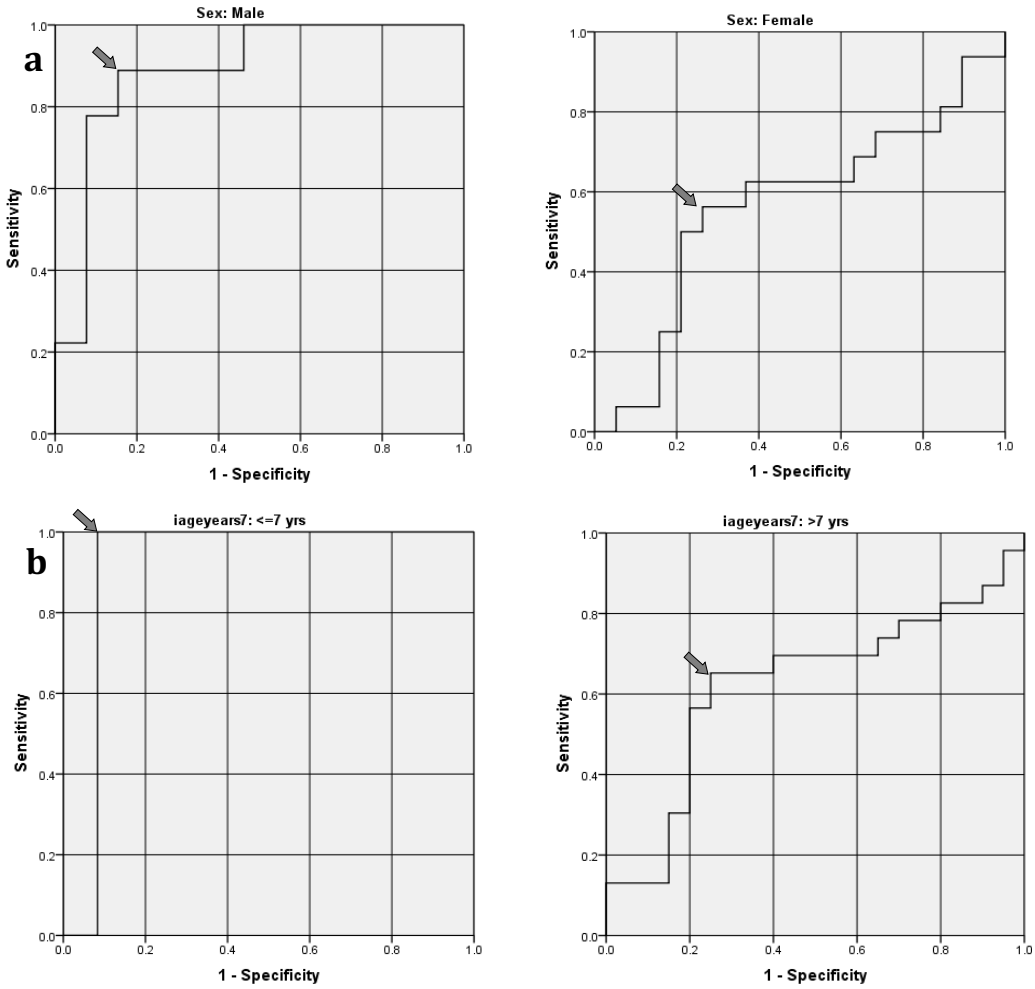

Table 5. Results of the ROC analysis of serum total LDH values in differentiating AL from IBD cats stratified according to gender and age.

Factors tested	Accuracy (AUC±SE)	Max LR	Best cut-off	Sensitivity	Specificity	PPV	NPV
Gender:							
- Neutered males	0.889±0.074 (P=0.002)	6.55	231-285	8/9 (88.9%)	11/13 (84.6%)	8/10 (80.0%)	11/12 (91.7%)
- Spayed females	0.566±0.102 (P=0.508)	1.85	239-243	9/16 (56.3%)	14/19 (73.7%)	9/14 (64.3%)	14/21 (66.7%)
	P=0.010	-	-	-	-	-	-
Age:							
- Up to 7 years	0.917±0.080 (P=0.068)	23.0	289-349	2/2 (100%)	11/12 (91.7%)	2/3 (66.7%)	11/14 (100%)
- Over 7 years	0.624±0.089 (P=0.165)	2.35	239-243	15/23 (65.2%)	15/20 (75.0%)	15/20 (75.0%)	15/23 (65.2%)
	P=0.014	-	-	-	-	-	-

AUC: area under the curve; LR: likelihood ratio; NPV: negative predictive value; PPV: positive predictive value; ROC: receiver operating characteristics; SE: standard error

5. Discussion

Middle-aged and old cats are often addressed to the veterinary clinic due to the presence of gastrointestinal symptoms, such as weight loss, anorexia, vomiting and diarrhea. In these patients, the diagnostic plan is complex and frequently leads to GI biopsy in order to obtain histological samples for diagnosis. Both IBD and AL are very common pathological conditions in cats, and they present a comparable clinical course[14]. The first step in the clinical evaluation of these patients is non-invasive routine laboratory testing and abdominal ultrasound examination[14]. Despite the fact that moderate to severe wall thickening, together with a loss of layering, presents a poor specificity, it is a highly suggestive ultrasonographic finding of neoplastic lesions (such as high-grade lymphomas); on the other hand, the wall appearance is

similar among low grade lymphomas and IBD[25]. In fact, both IBD and AL are characterized by diffuse or segmental distribution in the small intestine, with the ultrasonographic features of bowel wall thickening due to the increase in the muscularis propria and preservation of the wall layers without mass formation[25]. Moreover, a normal US examination does not rule out AL or IBD. However, in our study, cats with thickening of the muscularis propria detected on US and mesenteric lymphadenopathy were more likely to have lymphoma than IBD, and this is in accordance with another recent study[26].

The measurement of LDH as an initial screening for differentiating patients with different cancers (i.e. not only for lymphoma) and patients with non-oncological diseases has been used in dogs, but has not produced convincing results[9]. On the other hand, serum LDH levels demonstrated a prognostic value in canine lymphoma recurrence[11] and an LDH increase was correlated with a worse prognosis in feline lymphoma, similar to the results observed in humans[13]. Therefore, assessing serum total LDH as a possible marker for AL may play an important role in differentiating between these two conditions in cats.

The results of our study showed a statistically significant correlation between high serum total LDH values and AL in cats. In fact AL cats had significantly higher serum total LDH values than IBD cats ($P = 0.017$), as well as a significantly ($P = 0.002$) higher frequency of cats with serum total LDH values higher than the upper reference limit was found in AL cats (n=16, 64.0%) compared to IBD cats (n=7, 21.9%).

In addition higher values of median total LDH near to the significant levels were found in cats with high grade AL and in those with B-lymphoma as well as a non significant higher frequency of abnormal LDH values was found in cats with severe IBD.

As far as the gender and age classes are concerned, the spayed AL females, had significantly lower serum total LDH values than neutered AL males, while no significant differences have been found for gender in IBD cats. On the other hand, serum total LDH values were significantly higher in older than in younger IBD cats but not in the AL cats. Moreover, significantly higher values of LDH in AL versus IBD cats have been found in neutered males while in cats up to 7 years of age the difference was only close to the significant level.

The diagnostic accuracy in differentiating these two groups of cats in the population was significant, showing moderate sensitivity (68.0%) and specificity (78.1%). Gender (P=0.010) and age (P=0.014) significantly conditioned the diagnostic accuracy; in fact, significant values of accuracy were only found in neutered males (88.9%; P=0.002) and in cats up to 7 years of age it was particulary high even if it resulted being close to the significant level (91.7%; P=0.068). In particular, sensitivity prevailed both in netered males (88.9%) and in younger cats (100%) and a good values of NPV in both groups were detected (91.7% in neutered males and 100% in cats up to 7 years of age). The best cut-off for differentiating between AL and IBD cats was identified in a serum total LDH value ranging 239-243 U/L.

It has been shown in the literature that cats over 7 years of age have an increased risk of tumors and that intact males and females appeared to have a decreased risk as compared with neutered patients, but this could be explained by the age difference among these patients as the older patients were more likely to be neutered[23]. We have only neutered cats in the study group and this is of course a limitation. Possible explanations of our findings might be that LDH levels are more sensitive to the presence of tumors in cats under 7 years of age since they have a lower probability of tumor. Despite these observations, the role that gender and age may play in differentiating AL from IBD cats needs to be analyzed in additional studies.

As far as the relationship between breed and risk of tumor is concerned, an increased risk in the Siamese breed has been found[23]. There were no Siamese cats in our population; therefore, a confirmation of the role of LDH in specific breeds is needed involving larger samples.

The main strength of the present study is the particular selection of cases made in a realworld setting according to a prospective protocol. Fixed previously-defined groups of AL and IBD cats were not studied but only those patients which, at the end of the blood and imaging screening for chronic GI symptoms, required biopsy in order to be diagnosed as having intestinal inflammation or lymphoma. Thus, the manifest cases of lymphoma, as well as those with other benign chronic enteropathies already suspected by means of the initial screening, were not included in the study in order to ensure that the results of the present paper could be reliably applied in clinical practice.

The main limitation of the present study was the scarce sample size available due to the strict selection criteria applied. In particular, this weakness prevented the

possibility of better identifying the factors which play an independent role in affecting the differentiation between AL and IBD using multivariate analysis. In addition, a larger sample size would also have allowed us to reduce the gray zone of the best cut-off values estimated in some subgroups of cats. Another limitation of the present study was the failure to include the histopathologic evaluation of the ileal bioptic specimens. New studies have now confirmed the need to collect both duodenal and ileal bioptic specimens in dogs and cats having diarrhea caused by intestinal inflammation (e.g., IBD) or tumour (e.g., lymphoma) in either the small or the large intestine[27]. Even though ileal biopsy yields important diagnostic information, ileoscopy is a more technically more demanding and time-consuming endoscopic procedure for many clinicians and, for this reason, the new endoscopic recommendations do not include the histopathologic review of ileal mucosal specimens in the initial screening for chronic GI symptoms[28]. Another limitation of the study is that the biopsy samples were analised by two patologists.

In conclusion, although abnormal serum total LDH values measured at the initial screening were significantly more frequent in AL cats than in IBD cats, this test had moderate diagnostic accuracy (68.6%) in differentiating between these two conditions in the overall population. On the other hand, our study showed a good accuracy (88,9%) of serum total LDH in differentiating between AL and IBD in neutered males and in cats up to 7 years of age (91,7%) even if the diagnostic utility of LDH may warrant additional studies because of the small size of these subsets of patients.

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CHAPTER V
STOMACH WALL EVALUATION USING HELICAL HYDRO-COMPUTED TOMOGRAPHY

Adapted from:

Terragni R, Vignoli M, Rossi F, Laganga P, Leone VF, Graham JP, Russo M, Saunders JH. Stomach wall evaluation using helical hydro-computed tomography. Veterinary Radiology & Ultrasound 2012;53:402-405.

1. Abstract

In helical hydro-computed tomography (HHCT), water is used as a neutral luminal contrast medium together with intravenous iodine contrast medium for the diagnosis and staging of human gastric neoplasia. We evaluated the feasibility of HHCT in 11 healthy animals (nine dogs and two cats). Adequate uniform gastric distension was obtained with 30 ml water/kg body weight. Fourteen client-owned dogs and four cats with suspected or diagnosed gastric neoplasia then underwent HHCT followed by intravenous contrast medium administration. Focal thickening with moderate contrast enhancement has been found in 10 dogs and 3 cats. The extent of the lesion was assessed easily in all these patients. Three dogs and one cat had a normal stomach wall. One dog had multifocal thickening of the antrum but no histopathologic diagnosis has been made. Helical hydro-CT, followed by intravenous contrast medium administration, is a simple technique for assessing the stomach wall.

2. Introduction

In humans, the diagnosis of gastric disorders is done by means of endoscopy and double-contrast radiographic studies, which enable the detection of small-sized lesions. However, these techniques only allow visualization of the mucosa, thus preventing the assessment of transmural and extraserosal extension of disease. Additionally, these techniques cannot detect metastases, which makes them unsuitable for tumor staging.[1] Therefore, transabdominal ultrasonography (US), computed tomography (CT), magnetic resonance (MRI) imaging and endoscopic ultrasound (EUS) are preferred for staging, mostly being used in combination, as none of these modalities alone is sufficiently accurate.[1, 2]

Standard CT examinations are limited in their usefulness for the diagnosis of gastric cancer due to the presence of gas and fluid artifacts and the difficulty of evaluating gastric wall thickening when the stomach is distended incompletely.[1] The accuracy of CT in the diagnosis and staging of gastric cancer improves when helical CT of the stomach is performed after the oral administration of water. This procedure, which is termed HHCT, is followed by the intravenous injection of contrast medium to enhance the image of the gastric wall.[1] In veterinary medicine, endoscopy and US are

most commonly used to evaluate gastric disorders.[3] Sonographically, wall thickening and wall layer identification are key features for the detection and characterization of gastric lesions, particularly to differentiate inflammatory and neoplastic lesions. Commonly, the stomach is empty during the US evaluation, which precludes accurate assessment of wall thickness.[4] Virtual endoscopy has not been widely assessed in companion animals and is not considered useful compared to conventional endoscopy.[5]

Our aims were to describe and optimize the technique for HHCT in dogs and cats and to evaluate the applicability of this technique in patients suspected of having a gastric tumour.

3. Materials and methods

Nine healthy female adult crossbreed dogs and two healthy female domestic shorthair (DSH) cats were studied to optimize the technique. Their body weight ranged from 5 to 45 kg for the dogs and 3.5–4 kg for the cats. All owners gave their oral informed consent for the enrollment of their pets into the study. Ethical approval was not required since the animal involved in the study were scanned for orthopedical disease. The animals were fasted for 15 h before the examination. They were anesthetized with intravenous diazepam, followed by intravenous propofol. After endotracheal intubation, anesthesia was maintained with isofluorane and oxygen

A single slice helical scanner has been used for the normal animal study*. All the animals were in sternal recumbency and scanned from the caudal border of the heart to the left kidney. The slice thickness was 3 mm and the pitch was 2:1. The animals were hyperventilated during the procedure to produce temporary apnea for the purpose of reducing motion artifacts. Four different volumes of lukewarm water (10, 20, 30, and 40 ml/kg) were given via a gastric tube, and CT images were acquired immediately after each volume was given. The whole procedure was performed over a period of 5 min to minimize gastric emptying between the different volume administrations. After the last water administration, the gastric tube was withdrawn partially and the CT study repeated after intravenous administration of 800 mg/kg of nonionic iodinated contrast medium with a power injector at 5 ml/s. Scanning was started about 30 s after the beginning of the contrast medium injection. All the studies

were reconstructed with 1 mm slice thickness and reviewed using soft tissue and bone algorithms.

After the study of the normal animals, 14 adult dogs and 4 adult cats underwent helical-hydro CT. These 18 patients were all undergoing staging of confirmed (*n* = 11) or suspected (*n* = 7) gastric neoplasia. They were each given 30 ml/kg of water via gastric tube, which was considered appropriate based on the study on the normal animals. The patients were imaged before and after the administration of intravenous contrast medium (800 mg/kg). Various scanners hsve been used, since this was a multicentric study. These included a single-slice CT* in seven dogs and one cat, a 16-slice multidetector scanner† in six dogs and three cats, and a 4-slice multidetector scanner‡ in one dog. The slice thickness was 1.2–3.0 mm, depending on the machine. All the images of the normal and clinical animals were reviewed by a board-certified radiologist (M.V.), who was unaware of the histopathologic diagnosis in patients for which it was available. The seven patients with suspected neoplasia underwent endoscopic biopsy (*n* = 6) or US-guided biopsy (*n* = 1) to confirm or to exclude the CT diagnosis.

4. Results

In the healthy dogs and cats, the stomach wall appeared thickened before water administration because the stomach was collapsed and the gastric lumen was poorly visible, if at all. After the administration of 10 ml/kg, there was insufficient distension of the gastric wall in all the dogs and cats. The fundus was slightly distended, but the body, pyloric antrum and pyloric canal were still collapsed, thus preventing evaluation of the wall. After 20 ml/kg, the gastric wall was thinner in all regions. However, in the pyloric part and at the junction between the body and the pylorus, pronounced stomach folds were still visible and in contact with each other. With a total volume of 30 ml/kg, gastric distension was satisfactory, even though small rugal folds were still visible in the pyloric region and at the body-pyloric junction. After 40 ml/kg, the stomach was distended and there were no rugal folds visible. All parts of the stomach could be assessed clearly with the 30 ml/kg and the 40 ml/kg distension. After intravenous administration of contrast medium, uniform moderate contrast enhancement of the gastric wall, especially of the mucosa, was observed (Figs. 1, 2A–

F, and 3A–F). However, it was not possible to differentiate the layers of the gastric wall either before or after intravenous administration of the contrast medium. The comparison between the reconstructed images showed that the bone algorithm, despite its better spatial resolution, did not add further information.

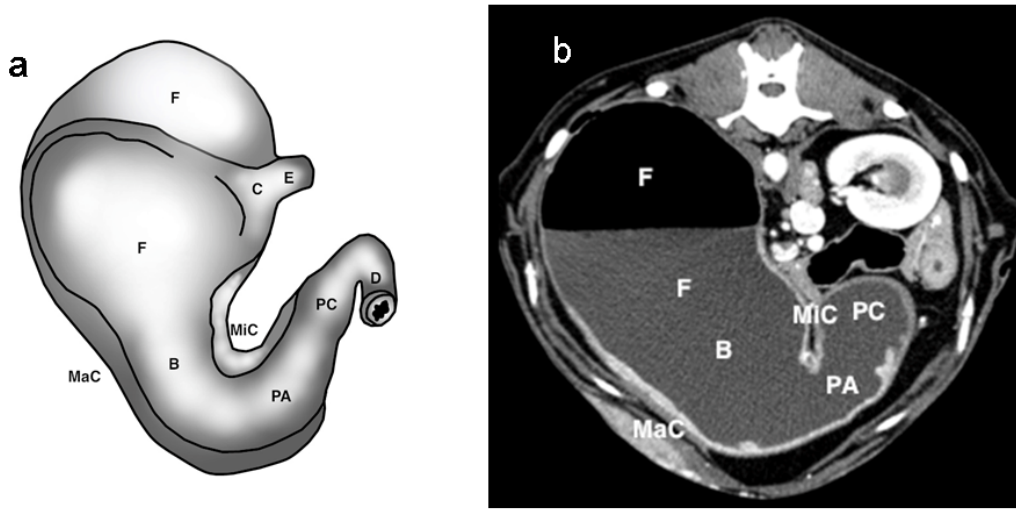

Fig 1. (A) Drawing of the stomach and (B) transverse CT image at the same level. B, body; C, cardia; D, duodenum; E, esophagus; F, fundus; MaC, major curvature; MiC, minor curvature; P, pylorus; PA, pyloric antrum; PC, pyloric canal.

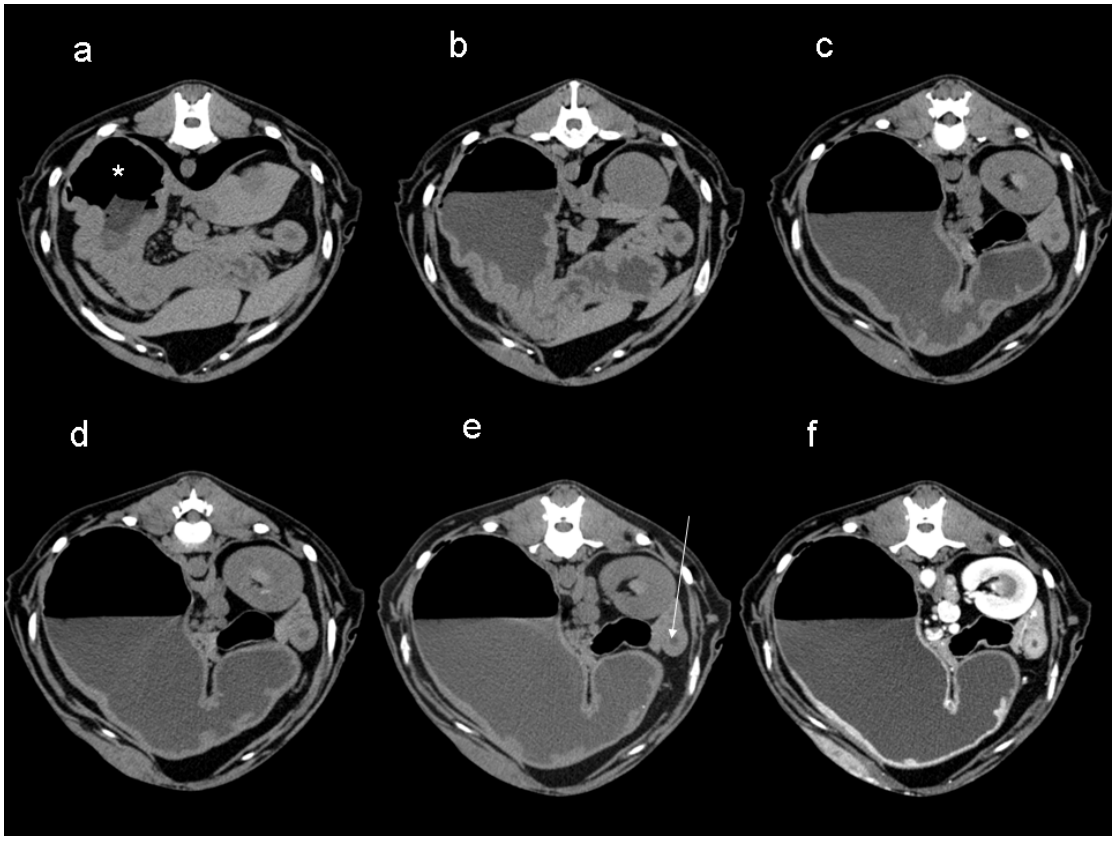

Fig 2. (A-F) Hydro-helical CT of a normal dog. (A) Native CT before administration of water. The fundus is filled partially with gas (asterisk) which allows evaluation of the wall at that location. The body, pyloric antrum and pyloric canal do not contain gas or fluid preventing adequate evaluation of the gastric wall. (B) After 10 ml/kg of water administration, only the proximal part of the fundus is distended adequately. The distal part of the fundus and the body have a small degree of distension but the rugal folds are still in contact. The other parts contain only a minimal amount of fluid. (C) After 20 ml/kg, the fundus and most part of the body are distended adequately. However, in the pyloric part and at the junction between body and pylorus, the distension does not allow optimal evaluation of the gastric wall. (D) After administration of 30 ml/kg of water, the degree of distension is satisfactory. Some folds are still visible in the body and pyloric parts of the stomach but they do not overlap. (E) After administration of 40 ml/kg of water, only a few folds are minimally still visible. A small amount of water is present in the duodenum (arrow). (F) After contrast medium administration there is moderate enhancement of the gastric mucosa.

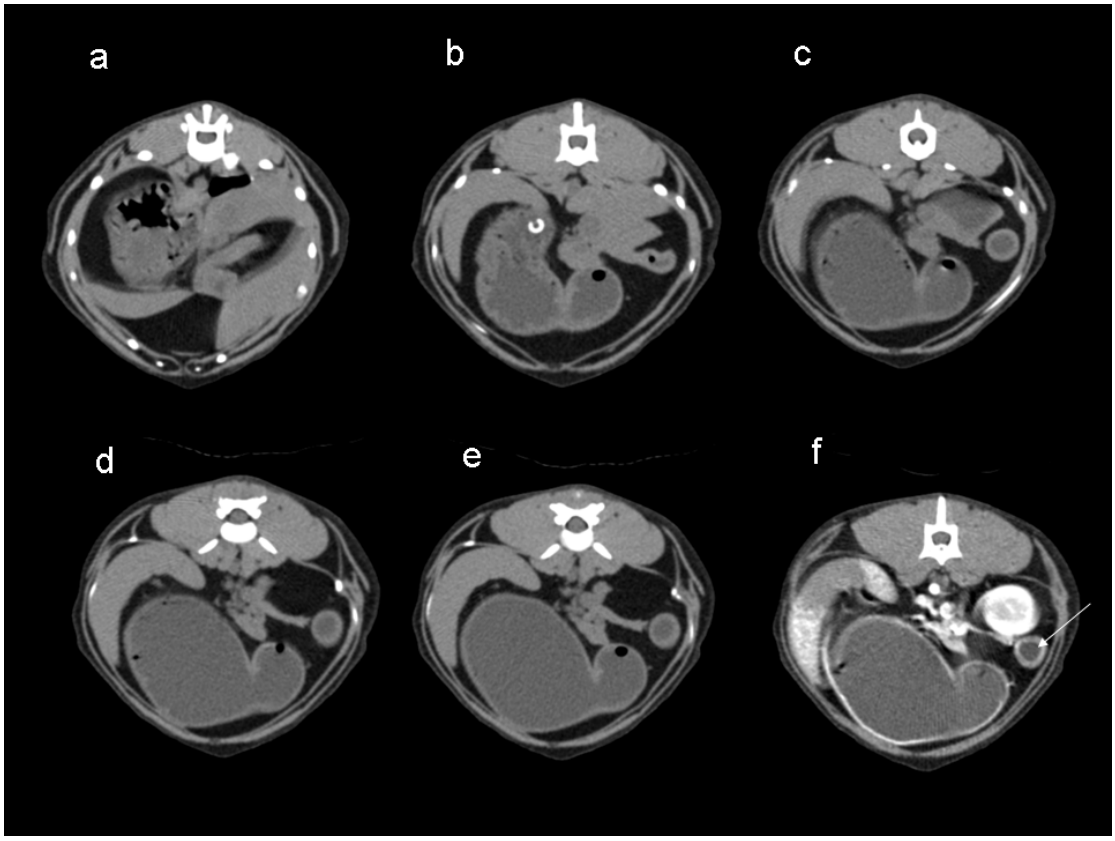

Fig 3. (A–F) Hydro-helical CT of a normal cat. As for the dog, the stomach wall evaluation was not possible with the stomach empty (A) or after administration of 10 ml/kg water (B) and suboptimal with 20 ml/kg water (C). Thirty milliliters per kilogram of water (D) was sufficient for diagnosis while with 40 ml/kg, all folds have disappeared. Figures (E) and (F) show the stomach after 40 ml/kg and plus contrast medium. Water within the duodenum is visible (arrow).

Fourteen of the 18 patients with either histologically diagnosed or suspected gastric neoplasia had an abnormal gastric wall with HHCT. Further, histopathologic evaluation revealed gastric adenocarcinoma in eight dogs, low-grade leyomyosarcoma in one dog, and lymphoma in one dog and three cats. One dog with an abnormal gastric wall on CT did not undergo biopsy because of the owner's refusal. Three dogs and one cat originally suspected of having a neoplastic lesion on US had a normal gastric wall on helical hydro-CT and neoplasia had been excluded on later histopathology.

In the eight dogs with adenocarcinoma, the CT findings corresponded to a focal, moderate gastric wall thickening with inhomogenous contrast enhancement and hyperattenuating mucosa. The lesions were in the lesser curvature in five dogs, in the pyloric antrum in two dogs and in the fundus/body in one dog (Fig. 4A and B).

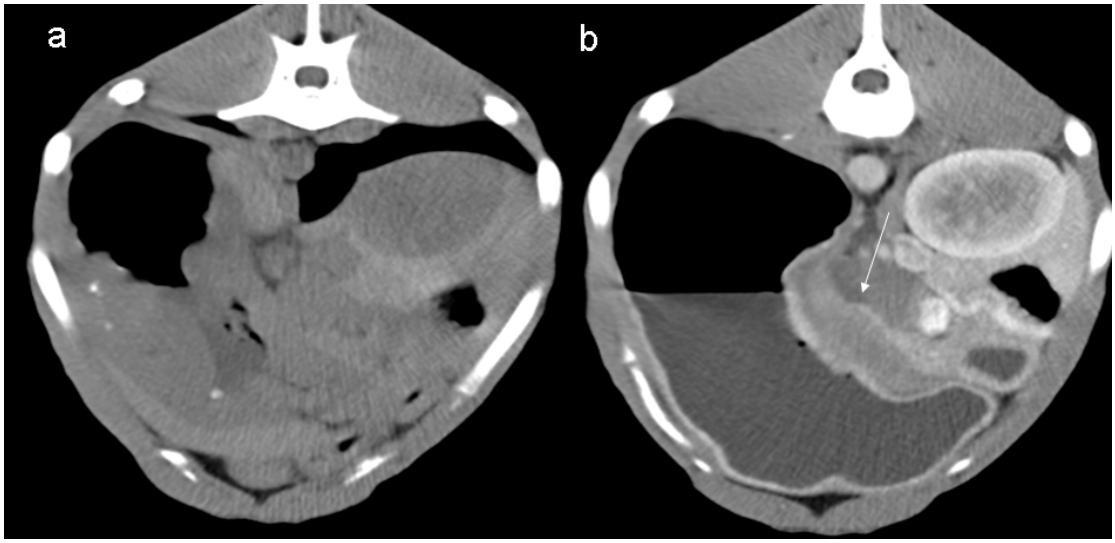

Fig 4. (A-B) CT studies of a dog with a gastric carcinoma: (A) CT scan with the stomach empty. There is a thickening of the gastric wall, especially at the level of the greater curvature at the body, and an irregular surface of the gastric mucosa of the lesser curvature of the body. (B) Same dog after distension with 30 ml water/kg and injection of intravenous contrast medium. The thickening of the lesser curvature at the body is clearly visible (arrows). The thickening of the gastric wall at the level of the lesser curvature of the body is clearly visible (arrow). There is moderate contrast enhancement.

The dog with a low-grade leiomyosarcoma had a discrete round mass filling almost all of the pyloric antrum and marked peripheral contrast enhancement. One dog with lymphoma had a moderate circumferential thickening of the gastric body and fundus with moderate inhomogenous contrast enhancement of the lesion and hyperattenuating mucosa. Of the three cats with lymphoma, one had moderate to severe gastric wall thickening at the cardias/fundus, another at the lesser curvature, and the third at the fundus/body (Fig. 5A–B).

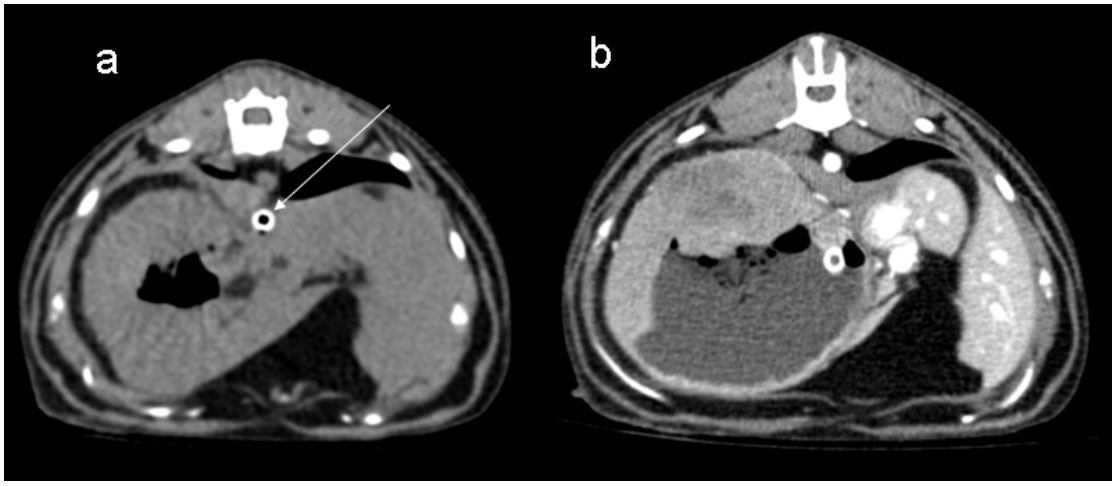

Fig 5. (A-B) Cat with gastric lymphoma. (A) Transverse image of an empty stomach– shows a circumferential thickening of the gastric wall. (B) After administration of 30 ml water/kg followed by intravenous contrast medium, a severe focal thickening of the greater curvature of the fundus and body with moderate contrast enhancement is visible. The gastric tube is visible (arrow).

All three had moderate heterogeneous contrast enhancement of the lesion. One cat also had a hiatal hernia (Fig. 6A–C).

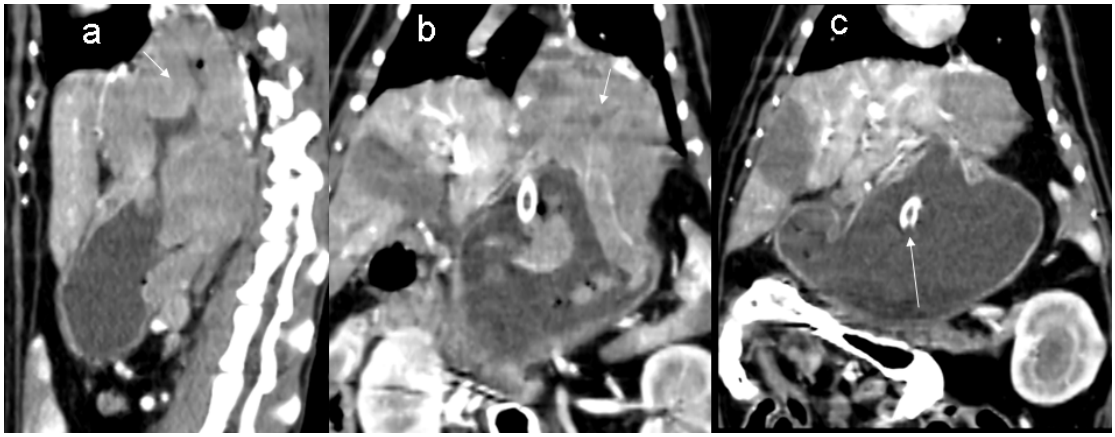

Fig 6. (A) Sagittal and (B,C) dorsal reconstruected CT images at different levels of the stomach in a cat with gastric lymphoma. Severe irregular thickening of the fundus with a hiatal hernia is present (short arrows). The enhancement of the gastric wall is heterogeneous and moderate. The gastric tube is visible (long arrow).

In the dog that did not undergo biopsies, a multifocal thickening at the pyloric antrum has been found, with marked contrast enhancement of the lesion and hyperattenuating mucosa.

Three dogs and one cat had a normal gastric wall on CT, thus excluding a neoplastic lesion. Multiple biopsies of the stomach wall have been taken, and histopathologic

evaluation confirmed the absence of neoplasia. In two dogs, the gastric lymph nodes were enlarged, and heterogeneous, and were found to be metastatic.
No adverse effects of the procedure were detected.

5. Discussion

Helical CT with water-filling of the gastric lumen followed by the administration of intravenous contrast medium has led to improvement in the detection and characterization of gastric cancer (GC) in humans[6] and is the method of choice for preoperative imaging of gastric carcinoma.[7] The main CT findings of gastric cancer are local or diffuse thickening of the gastric wall with variable contrast enhancement and an intraluminal soft tissue mass. Tumours can also infiltrate into perigastric tissue, encroach on adjacent organs and metastasize to distant sites.
We found that the wall of the normal collapsed, empty stomach appeared thickened before the administration of water, thus preventing the performance of reliable evaluation. After distension of the stomach with 30 ml water/kg body weight, all parts of the stomach could be satisfactorily assessed. The administration of intravenous contrast medium after the administration of water led to hyperattenuation of the mucosa in all normal dogs.
In the clinical patients, the thickening of the gastric wall on HHCT has been highly indicative of a gastric tumour in 14 of the 18 patients, and the absence of thickening excluded a tumour in 4 of the 18 patients. Seventeen of 18 patients had the diagnosis confirmed by histopathologic examination. Intravenous contrast studies in the clinical patients have been characterized mainly by heterogeneous contrast enhancement of the mass with a hyperattenuating mucosa. A specific pattern that would have enabled differentiation of the different types of gastric tumours has not been found.
The intravenous contrast study is mandatory for tumor staging to assess the presence of metastasis in lymph nodes and various other perigastric organs. In humans, the accuracy of HHCT is acceptable for staging of distant metastasis, but inadequate for staging lymph node metastasis of gastric carcinoma.[8] Helical hydro-CT also has a higher (86.0%) accuracy than that of conventional CT (72%) for staging GC.[6] In this study, two dogs had enlarged gastric lymph nodes that appeared to be metastatic on

cytologic examination. Nonetheless, HHCT is very useful for choosing between tumor resection and conservative treatment in patients with gastric tumours.

Helical hydro-CT can provide useful information about the location and extent of gastric lesions, as was shown in our study, and if surgery is an option. Helical hydro-CT is also valuable for excluding a gastric lesion suspected on other imaging modalities. In this study, three dogs and one cat suspected of having a gastric tumour on US had a normal stomach on HHCT. However, the US studies were performed without dilation of the stomach by water, which may explain the different interpretation. However, it is more relevant to compare native US with HHCT. The main advantage of US compared to CT is that it can be performed with no, or less, chemical restraint. Having to anesthetize the patient to administer water for US would clearly diminish the biggest advantage of this US in comparison with CT.

In veterinary medicine, the most frequent modalities used to study gastric lesions are US and endoscopy.[3, 4] Although we did not perform a direct comparison of these techniques with HHCT, we feel that HHCT could be superior to US and endoscopy in evaluating the location and local invasiveness of the lesion. On the other hand, US and endoscopy are probably superior in doing adequate biopsies.

Another possibility for dilating the stomach and studying the gastric wall more accurately with CT would be filling the stomach with air. However, this technique cannot be standardized because it is not possible to predict or measure the quantity of air that escapes from the stomach. Additionally, the use of air carries the theoretical risk of vascular embolization or gastric volvulus. With the use of water, no complications have been observed. Reasonable amounts of water simply pass through the stomach, as usual.

The images reviewed with a bone algorithm did not add further information, therefore it appears that a standard algorithm can be satisfactory for this technique.

In conclusion, HHCT is easy to perform and is useful for the diagnosis of gastric tumours in dogs and cats. The recommended dose of water is 30 ml/kg, and the HHCT study should be followed by the administration of intravenous contrast medium. Adequate distension of the stomach and optimally timed administration of intravenous contrast medium are mandatory to obtain high quality images and to detect and characterize the disease process.

*GE ProSpeed Power, Milwaukee, WI.

†BrightSpeed GE, Milwaukee WI.
‡Toshiba Aquilion, KS.

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CHAPTER VI

GENERAL DISCUSSION

The evaluation of clinico-pathological parameters and imaging diagnostics are the basis of the protocol in dogs and cats with gastric and intestinal tumors since they guide the clinician towards the diagnosis and the staging of a complex disease. Histopathologic diagnosis is the final aim of the diagnostic protocol but it is also the beginning of some important evaluations which influence therapy and prognosis.

In the first part of this study, 19 histopathological samples of gastric epithelial neoplasia in dogs have been analyzed: 5 adenomas and 14 carcinomas. Five (35.7%) carcinomas have been classified as intestinal and 9 (64.3%) as diffuse. They were retrospectively evaluated for EGFR/HER-2 immunohistochemical expression and KRAS mutational status. In human oncology, the importance of the EGFR/HER-2/KRAS signaling pathway in gastric cancer has been well established, and HER-2 testing is required before starting therapy. Conversely, this pathway has never been investigated in canine gastric tumours.

Gastric tumours account for <1% of all reported neoplasms in dogs.[1] Carcinoma is the most frequent type of gastric tumour, comprising 50-90% of all canine gastric malignancies and usually resulting in death.[2] The majority of canine gastric tumours are located in the lesser curvature and pylorus, often progressing to involve most of the stomach body.[2] The reported age range of dogs with gastric tumour is from 3 to 16 years of age (mean 7.5 years) and, as in humans, a higher incidence is reported in males.[3] Breed predisposition to gastric tumour in Belgian shepherd dogs (Tervuren and Groenendael), rough collies, Staffordshire terriers, chowchows and standard poodles has been reported.[4,5] Sled dogs (e.g., Alaskan malamutes, Siberian huskies, Pyrenean Mountain dogs) and Japanese akitas are often affected (unpublished data);[6] however, in determining any canine breed predisposition, the popularity of the breeds in the specific geographical location must be considered.

The incidence of gastric cancer is lower in dogs than in humans but, in recent years, this disease appears to have been diagnosed more frequently. This likely reflects the use of more accurate diagnostic techniques, such as endoscopy. Despite this, canine gastric tumours are often diagnosed in advanced stage, resulting in a poor prognosis and limited treatment options.[6] A median survival time of 35 days has been described and, in approximately 70-90% of cases, the tumours have already metastasized at the time of diagnosis or death.[6] Metastatic sites include the gastric lymph nodes, omentum, liver, duodenum, pancreas, spleen, oesophagus, adrenal glands and lungs.[6]

Most canine gastric tumours produce localized or diffuse thickening and/or ulceration of the gastric wall, serosal pallor and, occasionally, a substantial decrease in the number of rugae on imaging and gastroscopy.[7]

In humans, the development of gastric carcinoma often involves a sequence of events, starting with mild non-atrophic gastritis and progressing through stages of chronic atrophic gastritis, intestinal metaplasia, dysplasia and, ultimately, gastric adenocarcinoma.[8] In veterinary medicine, this sequential pathogenesis has not yet been defined, although metaplastic and dysplastic changes have also been recognized in the canine stomach. Canine gastric neoplasia has been well described and is now beginning to be explored at the molecular level. In human medicine, understanding the molecular events and pathways in gastric cancer has led to advances in prevention, early diagnosis, tumour classification and therapeutic intervention. A collections of genetic and molecular abnormalities occurring during gastric carcinogenesis has been described in detail, including the activation of oncogenes, overexpression of growth factors receptors, inactivation of tumour suppression genes, involvement of DNA repair genes and cell adhesion molecules, loss of heterogeneity and point mutations of tumour suppressor genes and the silencing of tumour suppressors.[9] In veterinary pathology, the first steps towards molecular studies are being taken. Reduced sampling, and the difficulty in validating and optimizing molecular biology techniques in animal tissues and samples could account for this delay. Nevertheless, some studies have been carried out in this area and often replicate the results which have been obtained in human pathology since strong similarities have been observed between dogs and humans with regard to the clinical presentation and the histopathological features of GC. Janke et al. tested 16 canine gastric tumours and found an overexpression of C2-OsLe(x) in 56% of the tumours when compared with normal gastric mucosa.[10] The authors hypothesized that these carbohydrates are highly expressed in more malignant types of canine gastric tumour; they promote adhesion to selectins and attachment of cancer cells to the vascular endothelium, and, therefore, metastasis.[10]

The type I growth factor receptor family consists of the prototype EGFR and the related members cerbB-2, c-erbB-3 and c-erbB-4 (also known as HER1, HER2, HER3 and HER4, respectively).[10] The membrane receptors have been shown to be important in the development of neoplasia.[10] The HER3 molecule differs from the other members of this family because it has little or no tyrosine kinase activity.[10] In

humans, the blockade of HER-3 activity decreased downstream signaling of the phosphoinositide 3-kinase/protein kinase B pathway and the cell cycle, resulting in the death of tumour cells. Previous investigations have suggested a close relationship between cytoplasmic/membrane HER3 expression and tumour prognosis for human gastric cancer. Doster et al. determined CDX2 (a specific marker for human cell adenocarcinoma) and HER3 expression in five canine gastric mucinous adenocarcinomas.[11] In all cases, strong cytoplasmic HER3 labelling has been recorded in >10% of the tumour cells, and the majority of the nuclei of the neoplastic cells showed strong CDX2 expression.[11] However, in this study, CDX2 and HER3 expression could not be correlated with prognosis due to the small population size and the lack of a complete clinical history and postsurgical follow-up.[11] A recent study in dogs focused on other members of this family; EGFR and its analogue HER2 and KRAS, which is a small protein encoded by the KRAS gene mediating the transduction of signals between EGFR receptors and the nucleus.[12] In human oncology, it has been shown that the KRAS gene can undergo mutation, the majority of which involve codons 12 and 13 identified as predictors of resistance to anti-EGFR drugs. Our study evaluated EGFR/HER2 immunoexpression and KRAS mutational status retrospectively in five adenomas and 14 carcinomas (5 intestinal and 9 diffuse carcinomas). Overexpression of EGFR and HER-2 has been observed in 42.1% and 57.9% of the cases, respectively, regardless of tumour location and biological behaviour. The percentage of EGFR-positive tumours was significantly higher in the intestinal type than in the diffuse type. Furthermore, the KRAS gene was of wild type in 18 cases while one infiltrative mucinous carcinoma of the gastric fundus with regional lymph node metastases harboured a point mutation at codon 12. This mutation is among the most frequently detected in humans and leads to the substitution of a glycine by an arginine, thus leading to a geometric alteration of the protein. This results in a lack of quanine triphosphate hydrolysis, which keeps KRAS in a permanently activated state.[13]

Genetic and/or epigenetic alterations in the E-cadherin-encoding gene (CDH1), or alterations in its protein expression, often result in tissue disorder, cellular de-differentiation, increased invasiveness of tumour cells and ultimately turns into metastasis.[13] In humans, CDH1 is regarded as a classical tumour suppressor gene in gastric carcinogenesis, being involved in the initiation and progression of both sporadic and hereditary forms. The potential role of E-cadherin (E-cad) and CDH1 in

canine GC has been investigated. Preliminary results show that the majority of canine gastric tumours have abnormal E-cad expression in comparison with the normal gastric mucosa. The different patterns of E-cad immunoexpression observed in canine GC are similar to those already described in human gastric cancer and the existence of CDH1 somatic alterations in such lesions may provide useful information for the clinical management and prognosis for these animals.[13] In another study, the expression of gastrin was investigated immunohistochemically in gastric biopsy samples from 64 dogs with gastric tumour. Only 8% of the canine carcinomas demonstrated expression of gastrin, which is considered less common than in humans. The authors concluded that this may not be a reliable prognostic marker in dogs.[14]

In the second part of this study, the diagnostic utility of measuring serum lactate dehydrogenase in cats with inflammatory bowel disease and alimentary lymphoma, which are common gastrointestinal diseases in cats, have been evaluated.

From literature it is known that, in cats with lymphoma, an increase in LDH is a negative prognostic survival factor associated with cellular necrosis.[15] In this study, serum LDH has been measured in a population of cats with the same chronic gastrointestinal symptoms.[15] AL and IBD are two pathological conditions which overlap from a symptological point of view and we aimed to measure the value of the enzyme and evaluate whether it can be useful in differentiating these two specific diseases.

In human primary gastric lymphoma, elevated LDH was associated with a poor outcome.[16] Both IBD and AL are very common pathological conditions in cats, and they present a comparable clinical course. The first step in the clinical evaluation of these patients is non-invasive routine laboratory testing and abdominal ultrasonograhic examination.[17] Despite the fact that moderate to severe wall thickening, together with a loss of layering, shows poor specificity, it is a highly suggestive US finding of neoplastic lesions (such as high-grade lymphomas); on the other hand, the wall appearance is similar among low grade lymphomas and IBD.[18] In fact, both IBD and AL are characterized by diffuse or segmental distribution in the small intestine, with the ultrasonographic features of bowel wall thickening due to the increase in the muscularis propria and the preservation of the wall layers without mass formation.[18] Moreover, a normal US examination does not rule out AL or IBD. However, in our study, cats with a thickening of the muscularis propria detected on

US and mesenteric lymphadenopathy were more likely to have lymphoma than IBD; this is in accordance with another recent study.[19] The measurement of LDH as initial screening for differentiating patients with different cancers (i.e. not only for lymphoma) and patients with non-oncological diseases has been used in dogs, but has not produced convincing results.[15] On the other hand, serum LDH levels have been demonstrated to have a prognostic value in canine lymphoma recurrence[20] and a LDH increase was correlated with a poorer prognosis in feline lymphoma, similar to the results observed in humans.[21] Therefore, assessing serum total LDH as a possible marker for AL may play an important role in differentiating between these two conditions in cats. The results of our study showed a significant correlation between high serum total LDH values and AL in cats; in fact, approximately 64% of the AL cats had abnormal LDH values as compared with 21,9 % of the IBD cats. In addition higher values of median total LDH close to the significant levels have been found in cats with high grade AL and in those with B-lymphoma as well as a non significant higher frequency of abnormal LDH values was found in cats with severe IBD. As far as the gender and age classes are concerned, the spayed AL females, had significantly lower serum total LDH values compared to neutered AL males, while no significant differences have been found for gender in IBD cats. On the other hand, serum total LDH values were significantly higher in older than in younger IBD cats but not in the AL cats. In addition, significantly higher values of LDH in AL versus IBD cats have been found in neutered males while in cats up to 7 years of age the difference was only close to the significant level. The diagnostic accuracy in differentiating these two groups of cats in the population was significant, showing moderate sensitivity (68.0%) and specificity (78.1%). Gender (P=0.010) and age (P=0.014) significantly conditioned the diagnostic accuracy; in fact, significant values of accuracy were only found in neutered males (88.9%; P=0.002) and in cats up to 7 years of age it was particulary high even if it resulted close to the significant level (91.7%; P=0.068). In particular, sensitivity prevailed both in netered males (88.9%) and in younger cats (100%) and a good values of NPV in both groups have been detected (91.7% in neutered males and 100% in cats up to 7 years of age).

Some authors have recently tested the use of supervised machine-learning algorithms to differentiate between the 2 diseases using data generated from noninvasive diagnostic tests. Complete blood count (CBC) and serum chemistry (SC) results for the following 3 groups of client-owned cats were analyzed: normal, IBD or AL.[22] For

GI diseases, and specifically for IBD and AL, previous studies have shown some common hematological and biochemical abnormalities. Hypoalbuminemia, the most common serum biochemical abnormality in cats with lymphoma, is thought to be a result of the disruption of intestinal wall integrity. Therefore, albumin is one possible analyte which could help to differentiate between IBD and AL. In previous studies, basic statistical analyses have shown laboratory differences between normal cats and cats with IBD and AL. Cats with IBD or AL tended to have mild non-regenerative anemia attributed to chronic disease and suppression of hematopoiesis as previously found. Few differences were identified as possible discriminators using traditional comparative statistics between the AL and IBD groups except for chloride and cholesterol. Prediction models using machine learning provided a method for differentiating between AL-IBD, AL-normal, and IBD-normal. These models can provide another non-invasive diagnostic tool to assist clinicians with differentiating between IBD and AL, and between diseased and non-diseased cats.[22]

The need for non-invasive markers for differentiating diseases from such an overlapping clinical presentation surely exists. Additional studies are necessary for an in-depth study of the subject. To date, the only thing known with certainty is that an elevated percentage of cats with clinical signs of chronic disease of the small intestine and ultrasonographic findings of thickening of the intestinal wall can have both chronic enteritis and an intestinal lymphoma.[23]

In the third part of this study, a diagnostic imaging method, namely HHCT has been studied.

Radiology without contrast medium is easily available but has scarce diagnostic value for gastrointestinal neoplasia. Using contrast medium certainly adds much more information but it is a complicated and time-consuming method. Abdominal ultrasonography and endoscopy are the most adequate imaging techniques for imaging gastric neoplasia in veterinary medicine.[24] Ultrasonography is non-invasive, and endoscopy is minimal invasive method that is of low risk for the patient. Besides for diagnosing gastric neoplasia, US is also used for studying lymph nodes and the intestines. Under US guidance, fine needle aspiration (FNA) of the tumour for cytological diagnosis is possible prior to endoscopy. However, US evaluation of the stomach can be limited due to the presence of gas or food within the lumen which create artifacts impeding thorough evaluation of the stomach wall.[24] The absence of

abnormal US findings does not exclude the presence of serious GI disease. Additionally, the ability to properly identify the correct location of a mass can be challenging when using US due to its dynamic nature and lack of consistent, well-defined anatomic landmarks.[26] Ultrasonographic examination of the stomach can be carried out by means of changing the position of the patient or by filling the stomach with a liquid, which is not easy to perform in veterinary medicine.

The US diagnosis must be confirmed by means of upper digestive endoscopy which permits direct visualization of the stomach. Undoubtedly, endoscopy is the best method for evaluating the gastric mucosa. The location of a gastric tumour is more easily assessed using endoscopy due to more established anatomic landmarks and the ability to visualize the entire luminal surface.[26] In a recent study, the authors compared the US and the endoscopic findings in a group of dogs and cats with histologically confirmed gastric neoplasia.[27] Endoscopy identified 95% gastric tumours compared to 50% for ultrasonography.[27] The agreement between the techniques for tumour location was only 36%.[27] Endoscopy is also the best method for obtaining biopsies for histopathological examination and permits the removal of pedunculated masses. Endoscopic biopsy is not an invasive procedure, requiring little time and is very adapted to critical patients; furthermore, it identifies the sites of the mucosa which are most suitable for biopsy. The results obtained from endoscopic biopsy depend on the experience of the operator and the equipment used.[28] However, ultrasonography is also operator-dependent. One disadvantage of endoscopy is that samples cannot be obtained from nonfasting patients and it is not always possible to reach the ileum, even for an expert operator. The "blind" biopsy technique of the ileum through the ileocolic sphincter is, however, possible. Some studies show a greater probability of diagnosing AL in cats by means of a full thicknesses laparatomic or laparoscopic biopsy respect to an endoscopic biopsy.[29,30] However, it can be said that comparative studies which document the superiority of one biopsy technique respect to another have not been published in the literature. Surely, specimens from many segments of the intestine increase the sensitivity of a diagnosis of AL and of other GI diseases. All thickness surgical biopsy is indicated if involvement of the submucosa/musculature is suspected or if the results obtained with the endoscopic biopsy are not correlated with the clinical results.[31] In another study evaluating endoscopic biopsies and full thickness biopsies in cats with AL and IBD, approximately one third of the cats with AL were incorrectly diagnosed with

endoscopic biopsies.[29] This limitation of endoscopic biopsy is due to the small tissue samples achievable with endoscopic bioptic forceps which are unable to reach the deeper layers of the stomach or intestinal wall.[32] If the lesion is identified on ultrasonographic examination, evaluation of the depth of the gastric wall changes may provide beneficial information before endoscopy and biopsy.[25,33,34]

The type of gastric neoplasia that is the most frequently missed by US is lymphoma. Five different small intestinal sonographic patterns of lymphoma distribution have been described in cats, including transmural-circumferential, transmural-segmental, transmural-nodular, transmural-bulky and mucosal infiltration.[34] In fact, the mucosal infiltration pattern can present a normal aspect of the gastric wall in the early phases of the disease due to the minimal mucosal thickening with normal parietal stratification.[35] This pattern may also produce a normal US image in the early stage of the disease and, therefore, it may not be identified on US examination.

A distinction in the US appearance of T-cell lymphoma in the small intestines has been made on the basis of thickening of the muscularis layer only.[18] Feline GI small cell T-cell lymphoma is becoming recognized as a distinct form of GI AL in cats, and appears similar to IBD on ultrasound and histology[18]. Both IBD and AL are characterized by diffuse or segmental distribution in the small intestine, with US features of bowel wall thickening owing to a diffuse circumferential thickening of the muscularis propria and preservation of wall layers without mass formation. In the latter study[18], the muscularis propria was twice as thick in the intestinal segments with lymphoma and IBD than in healthy cats, and was the major contributor to overall bowel wall thickening. A relationship between the thickness of the muscularis layer and the extent of the neoplastic lymphocytic infiltration has previously been described, with the muscularis thickening increasing the odds of transmural disease in the depth of the submucosa.[19] The ratio of the muscularis propria to the submucosa may serve as a useful marker for possible infiltration with lymphoma or IBD on US examination. Healthy cats had a ratio of <1 in the duodenum, and the majority of healthy cats had a ratio of <1 in the jejunum. In contrast, the ratio of the muscularis propria to the submucosa in the majority of cats affected with lymphoma was >1 and significantly higher in both segments. This measurement may be useful in identifying cats with muscularis propria thickening in addition to absolute measurements. Another study compared the wall layering of cats with IBD to those with intestinal AL, and found no US differences between the two diseases.[18] A previous study found

a stronger association between muscularis thickening and lymphoma than muscularis thickening and IBD when considering the prevalence in each population.[19] Both disease groups were clearly different from the population of healthy cats, and the ratio of muscularis propria to submucosa of >1 was indicative of small intestinal disease. Diffuse thickening of the muscularis layer of the small intestine with concurrent enlargement of the colic lymph nodes was associated with small cell T-cell GI lymphoma, although lymph node enlargement was not necessarily diagnostic for metastatic disease.[18]

When considering the computed tomography features of alimentary limphoma, in low-grade lymphomas there is less severe gastric wall thickening than in high-grade lymphoma, and abdominal lymphadenopathy is less common. The absence of abnormality or the presence of just minimal gastric wall thickening or a shallow lesion at CT suggests low-grade mucosa associated lymphoid tissue (MALT) lymphoma, subsequently CT is of limited value in its diagnosis. Hydro-helical CT (HHCT) refers to stomach CT with water distension. Compared to CT-gastrography with air distension, HHCT is believed to be less affected by artifacts caused by intraluminal air and is likely to demonstrate detailed mucosal enhancement of cancer foci. Traditionally, both air and tap water have been used as oral contrast agents to achieve adequate gastric distension for preoperative CT imaging in patients with early gastric cancer (EGC). CT is most frequently used for staging GC. Helical CT in combination with the water-filling method (HHCT) has led to marked improvement in the detection and characterization of gastric cancer. The main CT findings of GC are local or diffuse thickening of the gastric wall with variable enhancement and intraluminal soft tissue mass. The accuracy of HHCT (86.0%) in one study was higher than that of conventional CT (72%).[36] Tumour-node-metastasis (TNM) staging using HHCT depends on the improvement of CT equipment and stomach filling with water. Contrast-enhanced HHCT can additionally improve the accuracy of GC staging before surgery. In conclusion, the primary pathological condition of GC and its metastasis to adjacent or distant structures can be demonstrated by contrast-enhanced HHCT. HHCT can provide more information for surgeons to formulate an appropriate therapy for gastric cancer patients. A more recent study shows that, despite the introduction of multidetector row CT techniques and the use of multiplanar reconstruction (MPR) images, the detection rate of EGC on HHCT has still been unsatisfactory. Tumour size and invasion depth were independent factors for the

visibility of EGC on HHCT.[37] Double contrast radiography in humans is a overcome technique with the coming of endoscopy. In literature there're no comparison for diagnostic accuracy between contrast radiography and CT. To date, methods for more complex images, such as CT and MRI, have not been used for diagnosing gastrointestinal tumours in dogs and cats.

We evaluated the feasibility of HHCT in 11 healthy animals (nine dogs and two cats). Adequate uniform gastric distension was obtained with 30 ml water/kg body weight. After optimizing the technique, 14 dogs and 4 cats with suspected or diagnosed gastric neoplasia underwent HHCT followed by intravenous (IV) contrast medium administration. Focal thickening with moderate contrast enhancement of the wall was found in 10 dogs and 3 cats. The extent of the lesion was assessed easily in all the patients. HHCT, followed by IV contrast medium administration, is a simple technique for assessing the stomach wall in dogs and cats; it is easy to perform and is useful for diagnosing gastric tumours. The recommended dose of water is 30 ml/kg, and the hydro CT study should be followed by the administration of IV contrast medium. Adequate distension of the stomach and the optimally timed administration of intravenous contrast medium are mandatory for obtaining high quality images and for detecting and characterizing the disease process.

HHCT can provide useful information regarding the location and extent of gastric lesions, as was shown in our study, and whether surgery is an option. The technique is also valuable for excluding a suspected gastric lesion seen on other imaging modalities.

Future research studies

In recent years, a significant focus on the identification of biomarkers for early detection of gastrointestinal tumors has been observed in men. In gastric cancer conventional tumor markers do not allow diagnosis with adequate sensitivity and specificity; their use is limited to prognosis and follow-up recommendations determining the ideal treatment for an individual. However, several studies presently underway are attempting to establish new molecules as tumor markers. It is also well known that various genetic alterations, activation of oncogenes, inactivation of tumor suppressor genes, are necessary stages in gastric and intestinal cancer development.

In veterinary pathology the first steps towards molecular investigations are being taken. The reduced sampling and the difficulty in validating and optimizing molecular

biology techniques in animal tissues and samples could underlie this delay. Nevertheless, some studies have occurred in this area and often replicate results obtained in human pathology, like studies on CDX2, HER-3 and E-cadherin-encoding gene (CDH1). Strong similarities have been observed between both dogs and people with regard to the clinical presentation and histopathological features of GC. Future research study probably will be in the direction of identification of tumor biomarkers and molecular genetic behavior of these tumors.

Over the past two decades, advances in cross-sectional imaging, such as CT and MRI have dramatically changed the concept of GI imaging. Next to HHCT, these modalities offer additional diagnostic tools. CT-enterography performed after the oral administration of an isotonic solution in order to obtain adequate distension of the small bowel wall is the intestinal counterpart of HHCT and could provide a new insight in intestinal tumours. MRI has played a secondary role for years when compared to CT, especially due to the increased length of time of acquisition and the motion artifacts. However, the rapid development of the technical innovations, the introduction of new equipment and higher gradient magnetic fields have allowed the development of fast T1- and T2-weighted sequences with improved spatial and temporal resolution, single-shot fast spin-echo or gradient-echo, acquired during a single apnoea, which enabled the development of MRI protocols for studying the small bowel using an intraluminal contrast medium (MR-enterography). Finally, 18F-fluorodeoxyglucose PET/CT has proved to be useful in the management of gastro-entero-pancreatic tumours in humans and would be an interesting tool to study in veterinary medicine. Other new advanced imaging techniques, including magnification endoscopy, dye-based and dyeless chromoendoscopy and endomicroscopy provide real-time insights into the ultrastructural assessment of mucosal inflammation and dysplasia in humans.

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SUMMARY

Summary

Gastrointestinal (GI) neoplasia is not common in dogs and cats, with gastric tumours representing <1% and intestinal tumours <10% of overall neoplasia in these species. Gastrointestinal neoplasia tends to be malignant. The average age of dogs with GI neoplasia is 6–9 yr and of cats 10–12 yr. Adenocarcinomas are the most common GI neoplasia in dogs; lymphoma is the most common feline GI neoplasia. Early diagnosis is important as it may improve prognosis and life quality, nevertheless timely detection remains challenging in current veterinary practice. Therefore, the development of new methods to improve the diagnosis, prognostic capabilities or treatment approach is essential. Over-expression of "oncogenes", increased enzymatic activity and new imaging methods have been explored to provide an improved approach of GI tumours.

In **Chapter I**, the most common GI tumours are described, based on their clinical presentation and histological types. The clinico-pathological parameters used for diagnosis are briefly discussed and compared to their use in human medicine. Finally, the current imaging methods for diagnosis of GI tumours are described. For the moment, ultrasonography (US) is the most used imaging modality. Depending on the localization of the pathological process, it can prove useful to combine US with endoscopy. These modalities have progressively replaced the labour-intensive radiographic contrast studies. Computed tomography (CT) and MRI can also deliver useful information, but their use is currently limited compared to US and endoscopy.

The scientific aims of this thesis are presented in **Chapter II**. The final goal of this work is to provide new insights for the diagnosis of GI tumours in dogs and cats. In the first part of this thesis, the role of a protein tyrosine kinase erbB-2, frequently called HER2 - as a biomarker for GI tumours is studied. In the second part, the enzymatical activity of LDH in tumours versus in inflammatory processes is studied. In the third part, the feasibility, use and capabilities of a new imaging method, helical hydro-computed tomography (HHCT) are assessed.

The importance of the EGFR/HER-2/KRAS signaling pathway in gastric tumours is investigated in **Chapter III**. Canine gastric epithelial neoplasms (5 adenomas and 14 carcinomas) were retrospectively assessed for EGFR/HER-2 immunohistochemical expression and KRAS mutational status. A high expression of EGFR and HER-2 in

canine gastric epithelial tumours, suggesting a role of these receptors in carcinogenesis, especially when compared to the persistent negativity of normal gastric mucosa, was noted. Additionally, there was a significantly higher percentage of EGFR positive cases among intestinal-type carcinomas. Unlike in humans, a relationship between marker expression and anatomical location or the biological behaviour of tumours has not been observed. Finally, a codon 12 mutation in the *KRAS* gene has been identified, equivalent to that found in human gastric carcinomas, suggesting that this altered pathway may also exert a role in the pathogenesis of gastric tumours in dogs.

The potential use of an elevation of the serum total LDH in cats to differentiate alimentary lymphoma (AL) from non-neoplastic GI disease, such as IBD, was analyzed in **Chapter IV.** A prospective non-randomized controlled study was carried out in three Italian private veterinary clinics. Fifty-seven client-owned cats (25 lymphomas, 32 IBD) have been used in a prospective non-randomized controlled study. Serum samples of total LDH analysis were measured. Although abnormal serum total LDH values were measured at the initial screening in cats with AL compared to IBD, this test had fair diagnostic accuracy in differentiating between these two conditions in the overall population. On the other hand, the study showed a good accuracy (P=0.001) of serum total LDH in differentiating between AL and IBD in neutered males, and in cats up to 7 years old the difference was close to the significant level (P=0.088).

In **Chapter V**, the technique of HHCT has been described and optimized for use in dogs and cats, and its applicability in patients suspected of having a gastric tumour has been assessed. Nine healthy female adult crossbreed dogs and two healthy female domestic shorthair (DSH) cats have been studied to optimize the technique. After the study on the normal animals, 14 adult dogs and 4 adult cats with confirmed (n=11) or suspected (n=7) gastric neoplasia underwent HHCT. We concluded that HHCT is easy to perform and is useful for the diagnosis of gastric tumours in dogs and cats. The recommended dose of water is 30 ml/kg, and the HHCT study should be followed by the administration of intravenous contrast medium. Adequate distension of the stomach and optimally timed administration of intravenous contrast medium are

necessary to obtain high quality images and to detect and characterize the disease process.

Chapter VI contains the general discussion and conclusions of this thesis. Despite some limitations, this study has demonstrated the potential use of new approaches in improving our diagnostic capabilities, providing new means for better prognosis and therapy in patients with GI tumours.

SAMENVATTING

Gastro-intestinale (GI) neoplasie komt maar zelden voor bij honden en katten, bij dewelke maagtumoren <1% en darmtumoren <10% van het totale aantal neoplasie gevallen uitmaken. GI neoplasie is maar al te vaak maligne. De gemiddelde leeftijd bij honden met GI neoplasie ligt op 6-9 jaar, en bij katten op 10-12 jaar. Adenocarcinomen zijn de meest voorkomende vorm van GI neoplasie bij honden; lymfomen komen het vaakst voor bij katten. Een vroege diagnose is essentieel, gezien het grote effect op prognose en levenskwaliteit, doch een tijdige opsporing blijkt moeilijk in de huidige diergeneeskundige praktijk. Bijgevolg zouden de over-expressie van de zogenaamde 'oncogenen', toegenomen enzymatische activiteit en nieuwe beeldvormingsmethoden grondig verkend moeten worden om een meer efficiënte benadering van GI tumoren te bereiken.

In **Hoofdstuk I**, worden de meest voorkomende gastro-intestinale tumoren beschreven, op basis van klinisch beeld en histologisch type. De klinisch-pathologische parameters die gebruikt worden voor de diagnose, worden kort besproken en vergeleken met hun rol in de humane geneeskunde. Tenslotte wordt een overzicht gegeven van de beeldvormingsmethoden die actueel ingeschakeld worden bij het stellen van de diagnose van gastro-intestinale tumoren. Momenteel is een combinatie van echografie en endoscopie, afhankelijk van de plaats van de tumor, de meest geschikte beeldvormingsmethode.
De nieuwe modaliteiten nemen de plaats in van de arbeidsintensieve radiografische contrastonderzoeken. CT en MRI kunnen eveneens nuttige informatie opleveren, maar hun gebruik blijft voorlopig beperkt.

In **Hoofdstuk II** worden de wetenschappelijke doelstellingen van dit werk voorgesteld. De uiteindelijke doelstelling van deze doctoraatsverhandeling is het verwerven van nieuwe inzichten met betrekking tot de diagnose van GI tumoren bij honden en katten.
In het eerste deel van dit werk, wordt de rol van een proteïne, Receptor tyrosine-proteïne kinase erbB-2, ook wel gekend als HER2, als mogelijke biomerker voor GI tumoren onder de loep genomen. In het tweede deel wordt de enzymatische activiteit van LDH in tumoren vergeleken met die in inflammatoire processen. In het derde deel worden de haalbaarheid, het nut en de mogelijkheden van een nieuwe beeldvormingsmethode, helicale hydro-CT beoordeeld.

Het belang van de EGFR/HER-2/KRAS signalisatie pathways bij maagtumoren, wordt onderzocht in **Hoofdstuk III**. Neoplasie van het maagepitheel bij honden (5 adenomen en 14 carcinomen) werd retrospectief beoordeeld voor EGFR/HER-2 immuunhistochemische expressie en KRAS mutatiestatus. Een hoge expressie van EGFR en HER-2 bij tumoren van het maagepitheel bij honden werd vastgesteld, wijzend op een mogelijke rol van deze receptoren in de carcogenese. Bijkomend werd een significant hoger percentage van EGFR-positieve gevallen gevonden bij intestinale carcinomen. In tegenstelling tot bij mensen, werd een relatie tussen de merker expressie en de anatomische locatie of het biologische gedrag van de tumoren niet geobserveerd. Tot slot werd een codon 12 mutatie in het KRAS-gen geïdentificeerd, gelijkaardig aan wat gevonden wordt bij humane maagcarcinomen, erop wijzend dat deze aangepaste pathway mogelijk een rol speelt in de pathogenese van maagkanker bij honden.

In **hoofdstuk IV** wordt het mogelijke gebruik onderzocht van een toename van het totale LDH in het serum bij katten om te differentiëren tussen GI lymfomen en niet-neoplastische GI aandoeningen, zoals IBD. Een prospectieve, niet-gerandomiseerde, gecontroleerde studie werd uitgevoerd in de reële setting van drie Italiaanse private diergeneeskundige klinieken. Eenenzeventig katten van cliënten (33 lymfomen, 38 IBD) werden gebruikt. Het totale LDH in het serum werd gemeten. Hoewel abnormale totale LDH serumwaarden werden gemeten bij de initiële screening van de katten met GI lymfoom vergeleken met IBD, vertoonde deze test een slechte diagnostische accuraatheid bij het differentiëren tussen beide condities in de populatie. Anderzijds, vertoonde de studie een goede accuraatheid (meer dan 80%) van thet totale LDH in het serum voor het differentiëren tussen GI lymfomen en IBD bij gecastreerde katers en katten tot 8 jaar oud, zelfs indien de diagnostische waarde van LDH bijkomende studies behoeft, gezien de geringe omvang van de steekproef.

In **Hoofdstuk V** wordt de techniek van helicale hydro-CT beschreven en geoptimaliseerd voor toepassing op honden en katten. De toepasbaarheid van deze techniek voor patiënten, verdacht van een maagtumor, wordt beoordeeld. Negen gezonde, vrouwelijke hondenkruisingen en twee gezonde, vrouwelijke kortharige

katten (DSH) werden hiervoor bestudeerd. Na de studie van de normale dieren, ondergingen 14 volwassen honden en 4 volwassen katten, met een bevestigde (n=11) of een verdenking van (n=7) maagneoplasie, een helicale hydro-CT. De conclusie was dat een helicale hydro-CT vrij eenvoudig uit te voeren is en nuttig kan zijn voor de diagnose van maagtumoren bij honden en katten. De aanbevolen dosis water is 30 ml/kg, en de helicale hydro-CT-studie dient gevolgd te worden door de intraveneuze toediening van een contrastmedium. Voldoende distentie van de maag en optimaal getimede intraveneuze toediening van het contrastmedium zijn vereist om hoogkwalitatieve beelden te bekomen en om het ziekteproces naar behoren te detecteren en typeren.

Hoofdstuk VI bevat de discussie en het besluit van dit werk. Ondanks enkele beperkingen, heeft deze studie het potentieel aangetoond van verschillende benaderingen om onze diagnostische capaciteiten te verbeteren, hetgeen nieuwe perspectieven biedt voor de prognose en therapie bij patiënten met GI tumoren.

CURRICULUM VITAE

Curriculum vitae

Rossella Terragni is born on February 24th, 1970, in Bologna, Italy. She graduated in Veterinary Medicine at the University of Bologna in 1996. In 2001, she became specialist in "Pathology and Clinic of Companion Animals- Gastroenterology" at the University of Milan. In 2006, Rossella received the Master degree in "Gastroenterology and Flexible Endoscopy in Companion Animals" at the University of Teramo. From 1997 to 2013, she was a partner in two veterinary clinics: Orologio Veterinary Clinic and Veterinary Oncology Center in Sasso Marconi. From 2013 to 2016, Rossella was freelance for endoscopy in Bologna and Modena and in November 2016, she opened the Petcare Veterinary Clinic in Bologna.

Her special interests lay in internal medicine (gastroenterology), cytology and endoscopy in dogs and cats.

Rossella Terragni is author or co-author of more than 30 scientific publications in international journals and an active as speaker at national or international veterinary scientific and educational events.

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