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Canine gastric pathology

Helicobacter spp. infection in dogs - an epidemiological and molecular study

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DECLARATION

The author of this thesis declares that, in accordance with "nº 2, alínea a, do Art.º 31º do Decreto-Lei nº 230/2009", afforded a major contribution to the design and technical execution of the work, interpretation of the results and manuscript preparation resulting in the following accepted and submitted articles:

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This thesis also includes unpublished results.

Don’t dive in just to test the waters…
Do it to make waves!

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Nuna… I hope someday you'd be proud of your mummy.

And daddy… se tu estivesses aqui... sei que estarias feliz.

PREFACE

It was just another busy day at the microscope… Prof Fátima Gärtner and I were calmly performing the routine diagnostic examination in that small, and often too warm, room in the old building of ICBAS where the multiheaded microscope was located. I remember we were delightfully examining a gastric biopsy of a cat, in which small organisms with the "shape of a telephone wire" were stacked in the superficial mucus. We were, as they say, "enjoying" that perfect pink tinted image of haematoxylin and eosin stain, in which two different worlds were colliding…

Those small bacteria seemed to cling so strongly to the cell, just as a person hanging from a window would cling to the window sill... and it seemed that in that small instant, from a simple gesture, important decisions were being made! Just as a person hanging from the window sill must choose between letting go and falling into the abyss or swing back to the safety of the margin… It appeared that those bacteria were also struggling with a similar decision on that "precipice"... Climb up or not climb? Colonize or not colonize? Infect or not infect?

I guess pathologists are like that. They can spend hours contemplating a simple image that is both immensely complex and seductively beautiful. And they can create a whole background story, a whole movie script if you will, from that small instant.

And it was here that it all began… Before I knew it, I was completely enthralled in this mission.

Throughout the course of my doctoral studies, I had the luck of being able to choose my subject matter and the privilege of doing something that I love and something that completes me. Of course there were some not so happy moments….quite a few in fact! When we are in the necropsy room, we are confronted with a lifeless corpse, the smell is rotten, and there are no feelings. As we "stir" the cold innards, the work is serious and tranquil. The void of emotion is filled by the enthusiasm to discover and learn, by the enthusiasm of the students, by my enthusiasm of their enthusiasm.

The necropsies in the kennel were not like this. There, there are remnants of life. Your hands shift through once pulsating organs that are still warm. The intense smell of blood seems to penetrate and…..the sensation is different! One's concentration is diminished and one tries to hurry the process because of the "waiting line." We try to be professional but we can feel for the waiting animals whose destiny is one of silence. They can sense it too… They know what will happen next. There are barks and groans in the background and our heart breaks.

No, I have not yet had many experiences where I have been surrounded by students in the necropsy room, but I have had enough in the kennel to appreciate the difference. And this was, without a doubt, the most difficult part of the job.

Everything else was undertaken with great passion. And even after re-reading these last few paragraphs I must admit that, during this journey, I truly had a lot of fun!

RESUMO

Desde a descoberta do *Helicobacter pylori* (*H. pylori*) no início dos anos oitenta e da sua associação com patologia gástrica, a pesquisa científica voltada para as bactérias pertencentes ao género *Helicobacter* aumentou consideravelmente. Atualmente sabe-se que, para além de *H. pylori*, outras bactérias pertencentes ao mesmo género e comummente denominadas non-*H. pylori Helicobacters (NHPH)* podem colonizar e causar doença gástrica no Homem.

NHPH têm uma importância zoonótica e o cão é considerado um reservatório natural para muitas destas espécies. As espécies de *Helicobacter* que podem colonizar o cão são: *H. felis*, *H. bizzozeronii*, *H. heilmannii sensu stricto*, *H. salomonis*, *H. cynogastricus* e *H. baculiformis*.

Apesar da reconhecida importância do *H. pylori* no Homem enquanto causa primária de gastrite crónica, úlcera péptica, carcinoma e linfoma gástrico, o significado patogénico relacionado com a presença de *Helicobacter* no estômago canino é polémico.

O presente trabalho constitui um importante contributo para a compreensão das variadas patologias gástricas que podem afectar o cão. Um particular enfoque é atribuído à infecção por *Helicobacter* e aos mecanismos moleculares subjacentes à adesão das várias espécies à mucosa gástrica canina.

Aqui é descrito pela primeira vez o perfil de glicosilação do estômago canino e a sua influência na capacidade de adesão das diferentes NHPH à mucosa gástrica do cão, através do recurso a ensaios *in vitro*. Para além disso, a prevalência das NHPH no estômago de cães, na nossa região geográfica, foi estudada e as respectivas alterações histopatológicas associadas foram avaliadas.

Daqui se conclui que as diferenças no perfil de glicosilação da mucosa gástrica canina intervêm no processo de adaptação bactéria/hospedeiro e são sugestivas de que a adesão à mucosa gástrica pelos NHPH é mediada por mecanismos diferentes daqueles envolvidos na adesão do *H. pylori* à mucosa gátsrica humana e que até à data se desconhecem. Apesar da elevada incidência e distribuição mundial das NHPH, a prevalência de uma determinada espécie no estômago canino está também dependente de considerações geográficas.

ABSTRACT

Since the discovery of *Helicobacter pylori* in the early eighties and its association with gastric pathology, research on the *Helicobacter* genus has increased tremendously. In addition to *H. pylori*, other helicobacters commonly denominated non-*H. pylori Helicobacters* (NHPH) are found to colonize the human stomach and have been associated with gastric disease in humans.

NHPH are of zoonotic significance and the dog remains a natural reservoir for many species. The predominanst gastric *Helicobacter spp.* in dogs are *H. felis*, *H. bizzozeronii* and *H. heilmannii sensu stricto*, *H. salomonis*, *H. cynogastricus* and *H. baculiformis*.

Although *H. pylori* is well accepted as an important human pathogen and is believed to be the primary cause of chronic gastritis, gastric and duodenal ulceration, and even gastric carcinoma and mucosal-associated lymphoid tissue lymphoma, the pathogenic significance of gastric *Helicobacter* species in dogs is controversial.

This study provides an important contribution to the understanding of the gastric pathological disorders that can affect the dog. Additionally, a special focus is given to the *Helicobacter spp.* infection and to the molecular mechanisms underlying dog-related *Helicobacter* species adhesion.

Herein, it was described for the first time the glycosylation profile of the canine stomach and the *in vitro* binding capacity of FITC-labelled *H. pylori* and NHPH in cases representative of the canine gastric glycosylation pattern was tested. Furthermore, the prevalence of NHPH in the stomach of dogs within our geographical location and the associated histopathological changes were assessed.

With the present investigation it was concluded that the differences in canine gastric mucosa glycosylation profile play a role in the host adaptation of the gastric *Helicobacter* species and indicate that adhesion of NHPH involves different mechanisms, probably mediated by proteins with alternative receptor specificity yet to be characterized. Furthermore, despite the high prevalence and worldwide distribution of NHPH, geographic variations partially account for the prevalence of a specific *Helicobacter* species in the dog stomach.

ABREVIATIONS

Alp - Adherence-associated lipoprotein
APUD – Amine precursor uptake decarboxylase
ATPase - Adenosine triphosphatase
α - Alpha
Bab - Blood group antigen-binding adhesin
bp - Base pairs
BMP – Bone morphogenetic protein
β - Beta
cag PAI - cag pathogenicity island
Cag - Cytotoxin-associated protein
COX – Cyclooxygenase
CSA – Canine secretory alloantigen alloantibody system
DAB - Diaminobenzidine tetrahydrochloride
DBA – *Dolichos biflorus* agglutinin
DEA – Dog erythrocyte antigen
DNA - Desoxyribonucleic acid
dNTP - Deoxyribonucleotide triphosphate
E-cad – Epithelial cadherin
Fla – Flagellin
FITC – Fluorescein isothiocyanate
Fuc - Fucose
FUT - Fucosyltransferase
γ - Gama
GC – Gastric cancer
GD - Globoid dysplasia
GI - Gastrointestinal
GP – Gastric polyp
h – Hour
HCl - Hydrochloric acid
HE - Haematoxylin and eosin
H. – Helicobacter
H. americana - Heterobilharzia americana
HtrA – high temperature requirement
IARC - International Agency for Research on Cancer

IFN - Interferon
Ig - Immunoglobulin
IL - Interleukin
IM – Intestinal metaplasia
K - Potassium
Le - Lewis antigen
LPS - Lipopolysaccharide
M - Molar
MALT - Mucosa-associated lymphoid tissue
MCP – Methyl-accepting chemotaxis proteins
MG – Modified Giemsa
min - Minute
ml – Milliliter
MUC – Mucin
µM - Micromolar
µl - Microliter
Na - Sodium
NA – Not applicable
NAP - neutrophil-activating protein
NHPH - Non-*Helicobacter pylori Helicobacter*
ND - Not determined
NSAID - Non-steroidal anti-inflammatory drug
Oip - Outer inflammatory protein
OMP - Outer membrane protein
PAS - Periodic acid schiff
PCR - Polymerase chain reaction
RBC – Red blood cells
RER - Rough endoplasmic reticulum
RNA - Ribonucleic acid
Sab - Sialic acid binding adhesin
Ser - serine
Th - T-helper
Thr - Threonine
Vac - Vacuolating cytotoxin
VIP – Vasoactive intestinal polypeptide
WSAVA – World Small Animal Veterinary Association
WHO – World Health Organization
U - Unit

TABLE OF CONTENTS

CHAPTER 1 ..... **1**
General Introduction
- Histology of the stomach **5**
- Glycophenotype of gastric mucosa **10**
- Gastric *Helicobacter* species in humans and dogs **17**
- Gastric *Helicobacter* species related disease in humans and dogs **19**
- Pathogenesis of *Helicobacter* infections **22**

References **29**
Paper I: Unravelling canine gastric pathology **41**
Aims and Objectives **94**

CHAPTER 2 ..... **97**
***Helicobacter spp.* adhesion to canine gastric mucosa**
Paper II: A comparison of *Helicobacter pylori* and *non-Helicobacter pylori Helicobacter spp.* binding to canine gastric mucosa with defined gastric glycophenotype **99**

CHAPTER 3 ..... **113**
Epidemiological characterization of *Helicobacter spp.* present in the canine stomach
Paper III: Epidemiological characterization of *Helicobacter spp.* present in the canine stomach **115**

CHAPTER 4 ..... **133**
Molecular features of canine gastric lesions
Paper IV: An immunohistochemical study of canine spontaneous gastric polyps **135**

CHAPTER 5 ..... **143**
General Discussion **145**
References **152**
Summary and Conclusions **156**
Future Work and Perspectives **158**

APPENDIX **159**

- General Introduction

- Aim and Objectives

CHAPTER 1

The original illustrations of this chapter were created by the students David Lopes and Hugo Fernandes from the Faculdade de Belas Artes da Universidade do Porto (FBAUP-UP) and by Dr. Carlos Diogo Paulo, researcher at the Institute of Pathology and Molecular Immunology of the University of Porto (IPATIMUP).

Chapter 1

General Introduction

- Histology of the stomach
- Glycophenotype of gastric mucosa
- Gastric *Helicobacter* species in humans and dogs
- Gastric *Helicobacter* species and associated disease in humans and dogs
- Pathogenesis of *Helicobacter* infections

Paper I

- Unravelling canine gastric pathlogic

Aim and Objectives

- Histology of the stomach

The stomach is an expanded organ of the digestive system located between the oesophagus and the small intestine. Four different anatomical regions are identified in the stomach: cardia, fundus, body and antrum (or pylorus) (Dellman and Eurell, 1998).

The cardia is a narrow band near the opening of the oesophagus. The fundus is the dome-shaped region of the stomach. The body accounts for the major portion and narrows distally as it merges with the antrum. The antrum is the distal part of the stomach and includes the pyloric sphincter (Wilcock, 2013) (**Figure 1**). The anatomic boundaries separating these regions are not macroscopically distinct, at least not in the unopened specimen.

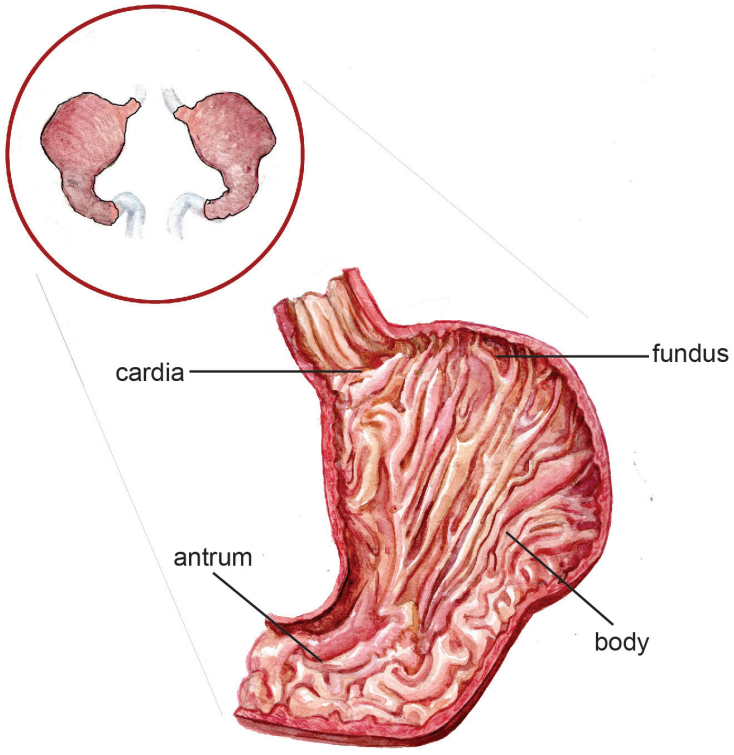


Figure 1 – Schematic representation of the different stomach regions.

(Illustration by David Lopes)

Grossly, the stomach presents a number of folds or ridges, called rugae, formed by the submucosa and mucosa, which serve to accommodate the filling and expanding of the stomach.

The stomach wall is composed of layers. From inside to outside, the first main layer is the mucosa. This consists of an overlying epithelium, supported by the *lamina propria* and underneath a thin smooth muscle layer called the *muscularis mucosae*. The second layer is the submucosa composed of dense irregular connective tissue. The following smooth muscle layer consists of a circular and longitudinal part lined by the serosa (Dellman and Eurell, 1998).

The fundus and the body present identical microscopic structure and therefore only three regions are histologically considered. The other gastric regions are histologically distinct and are mainly characterized according to the nature of the glands present (Junqueira and Carneiro, 2005).

Histologically, the surface of the stomach is lined by a simple columnar epithelium whose cells are called ***surface mucous cells***. These produce a cloudy, viscous and alkaline mucus that forms a thick gel-like coat that adheres to the surface epithelium.

There are numerous invaginations of the surface epithelium into the *lamina propria*. These invaginations are called gastric pits or foveolae and are also lined by surface mucous cells.

The cardia region contains cardiac glands which are heavily branched tubular structures composed almost entirely of mucous-secreting cells, with the odd neuroendocrine cells (described below) present. Their secretion protects the oesophagus against gastric reflux. Gastric pits in the cardiac region are fairly shallow (Wilcock, 2013).

The gastric or fundic glands present in the fundic region extend all the way to the *muscularis mucosae*. In this region, almost the entire *lamina propria* is occupied by glands. The lumina of the glands are usually not identifiable and they usually appear more like cords of cells. The only “typical” *lamina propria* can be seen in the areas between the foveolae and around the bases of the glands **(Figure 2)**. The following cells types can be seen in the glands of the fundic region: mucous-neck cells, parietal (or oxyntic), chief (zymogenic) and neuroendocrine cells (Dellman and Eurell, 1998; Junqueira and Carneiro, 2005) (**Figure 3**) .

The ***mucous neck cells*** are located in the neck region and secrete a soluble mucous only under vagal stimulation (not in the resting stomach). They have an irregular shape, with nuclei placed at the basal pole of the cell and the secretory granules close to the apical surface. The type of secreted mucin is different from that of the surface mucous epithelial cells (Junqueira and Carneiro, 2005).

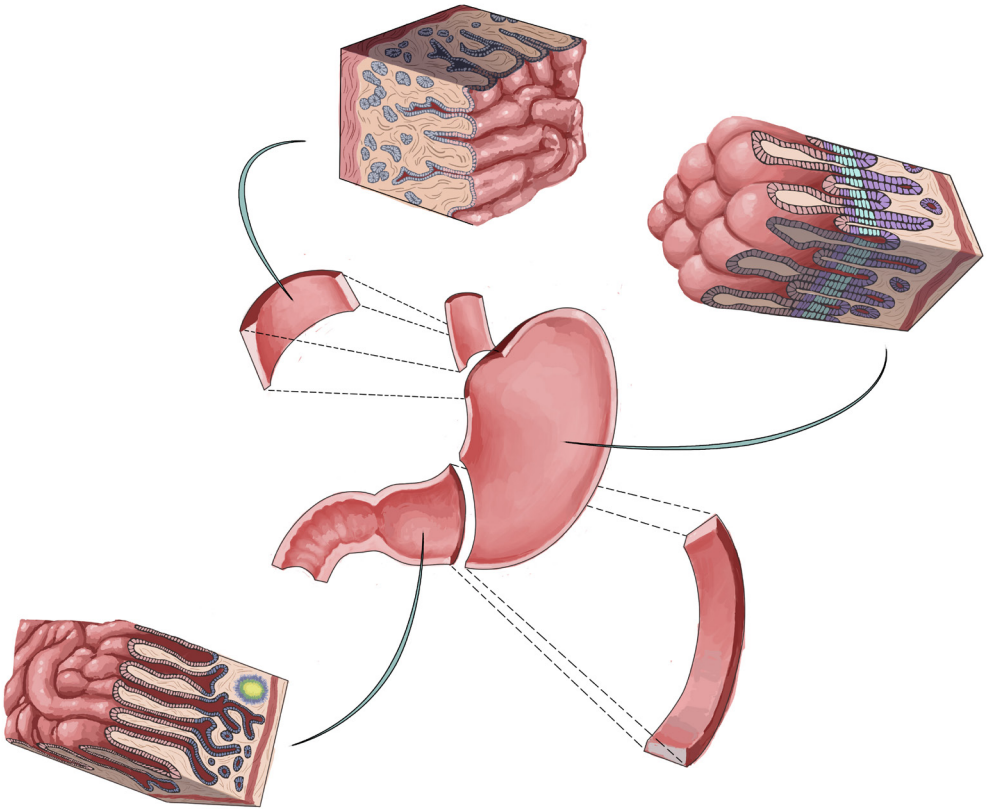


Figure 2 – Schematic representation of the anatomy and histology of the carnivorous stomach.
(Illustration by Hugo Fernandes).

Parietal (or oxynctic) cells are found predominantly in the upper part of the gland interspersed among the mucous neck cells. They are large in size, rounded or pyramidal, present spherical nucleus that occupies the central position and cytoplasm intensely eosinophilic due to the presence of numerous mitochondria and of extensive intracellular canaliculi (Junqueira and Carneiro, 2005). The latter remain important structures in the mechanism of hydrochloric acid production as parietal cells secrete the hydrochloric acid (HCl) of the gastric juice and intrinsic factor (a glycoprotein that binds to vitamin B12,

essential for maturation of red blood cells) (Dellman and Eurell, 1998).

Chief (zymogenic) cells are located mainly in the distal portion of the fundic glands and are typical-looking protein-secreting cells. Their basophilia stems from their abundant rough endoplasmic reticulum (RER). They secrete pepsinogen and a weak lipase. Pepsinogen is converted to the proteolytic enzyme pepsin upon contact with the acidic gastric juice being responsible for the stomach's ability to initiate proteins digestion (Dellman and Eurell, 1998).

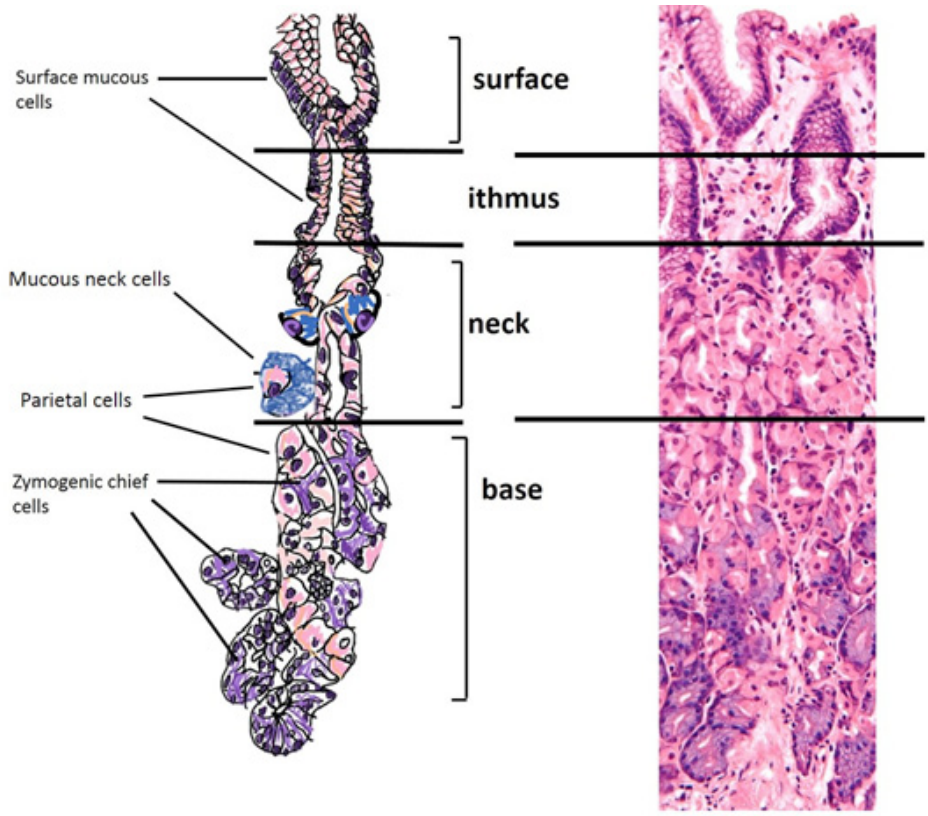


Figure 3 – Schematic representation of the cells types present in the fundic gastric glands. (Illustration by *Carlos Diogo Paulo*)

The ***neuroendocrine cells*** are specialized endocrine cells more prevalent near the base but can be found anywhere in the glands. They are not readily identifiable in standard histological preparations but may be highlighted recurring to silver stains, reason why they are also known as argentaffin and argyrophilic cells (Wilcock, 2013). Other names include enterochromaffin-*like* and amine precursor uptake decarboxylase (APUD) cells. Neuroendocrine cells secrete their product into the *lamina propria* whence it is taken up by blood vessels. Thus, stains that reveal their granules show them to be at the basal, rather than in the luminal surface. The major secretory product of these type of gastric cells is gastrin, which stimulates the production of HCl. Other products are glucagon, serotonin, substance P and vasoactive intestinal polypeptide (VIP) (Dellman and Eurell, 1998).

Other cells frequently found in the neck region that give rise to all the other cell types are called **undifferentiated (stem) cells**. They are low columnar cells, present high nucleous:cytoplasm ratio, lack of granules or mini-granules and are few in number. They are difficult to identify (their presence is revealed in preparations treated with tritiated thymidine). They travel upwards to replace surface mucous cells and downward to replace parietal, chief and neuroendocrine cells (Mills and Shivdasani, 2011).

The gastric antrum has few parietal or chief cells but has a separate population of alkaline, mucous-producing cells near the base of gland units that, in terms of molecular marker expression, resemble fundic or body mucous neck cells (Mills and Shivdasani, 2011).

- Glycophenotype of gastric mucosa

All cell surfaces, from all species ever studied, are decorated with a varied collection of glycans (Varki *et al.*, 2009). Together with nucleic acids, proteins and lipids, glycans constitute one of the four fundamental macromolecular components of cells that are involved in a multitude of intercellular and intracellular processes. Among all their complex functions, glycans are recognized targets for several viruses, bacteria and parasites mediating crucial host cell-pathogen interactions (Hooper and Gordon, 2001)

Glycans are covalently linked to other non-carbohydrates molecules forming glycoconjugates. Different glycans structures are assumed to exist in a mammalian cell glycome (Hart and Copeland, 2010). In nature, different families of glycoconjugates can be found, such as: glycosphingolipids, glycosaminoglycans, glycoproteins and glycosylphosphatidylinositol-linked proteins (Fuster and Esko, 2005).

Glycoproteins are glycoconjugates constituted by a protein that carries one or more oligosaccharide chains covalently attached to a polypeptide backbone, via N- or O- linkages (Varki *et al.*, 2009). This O-glycosylation in particular, is a stepwise process consisting in the addition of one monosaccharide at time, beginning with the addition of a sugar residue to either a serine (Ser) or threonine (Thr) amino acids (Varki *et al.*, 2009).

The biosynthesis of glycan chains is controlled by the enzymatic activity of several glycosyltransferases, which are expressed in a cell, tissue and species-specific maner. Various families of glycosyltransferases have been described and their activities and specificities characterized (Reis *et al.*, 2010; Varki *et al.*, 2009).

ABO(H) blood group system and Lewis antigens

The ABO blood group system was first identified by the Austrian immunologist Karl Landsteiner, in 1901. Landsteiner found different patterns of agglutination when mixing the serum and cells of all the researchers of his lab. Later it was clarified that what determined this agglutination was the antibody recognition of different glycan structures, the A, B and O antigens, expressed on red blood cells (RBC) surface. Three genes control the expression of ABO antigens: *ABO*, *Hh* and *Se* (Patenaude *et al.*, 2002) **(Table 1)**.

The *H* gene codes for the production of an enzyme transferase that attaches terminal α1,2-fucose to the RBC membrane anchored protein or lipid chain originating a precursor carbohydrate, called the H antigen, that is synthesize by all people. Specific enzymes synthesized by the *ABO* genes attach additional monosaccharides to the H antigen, and the final carbohydrate determines that person's blood type (Clausen and Hakomori, 1989).

Table 1 - Genes of the human ABO system

Gene	Gene product	Location
H (FUT1)	(α-1,2) fucosyltransferase (Fuc-TI)	19q13.3
Se (FUT2)	(α-1,2) fucosyltransferase (Fuc-TII)	19q13.3
A	(α-1,3) N-acetylgalactosaminyltransferase	9q34.1-q34.2
B	(α-1,3) Galactosyltransferase	9q34.1-q34.2

The *A* gene codifies for an α1,3GalNAcT and leads to the A antigen; *B*, codifies for α1,3GalT and leads to B antigen. If no A or B gene/enzyme is present, the H specificity remains, and the individual is of group O (Clausen and Hakomori, 1989) (**Figure 4**).

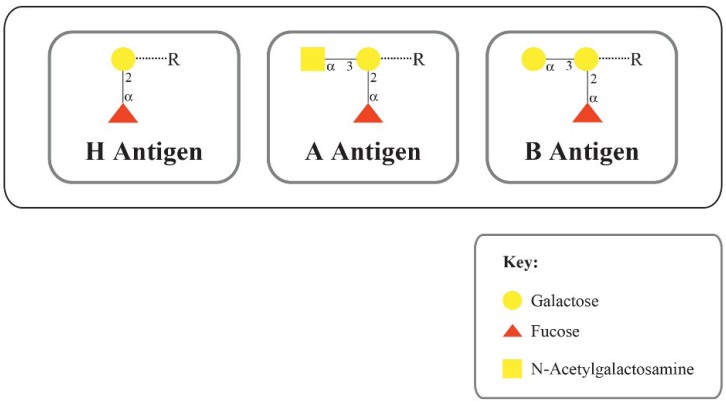


Figure 4 - Schematic representation of A, B and O(H) blood group structures.

Individuals from the blood group A, have AA or AO genotypes and express the antigen A. Individuals from the blood group B have a BB or BO genotype and express the B antigen. Individuals with the AB alleles belong to AB blood group and express both A and B antigens. Individuals which are unable to express any of these antigens, belong to the blood group O, and have OO genotype (Hosoi, 2008).

Different levels of expression of A (or B) on RBC are classified into subgroups. About 80% of group A individuals are A^1 and the remaining are A^2. It has been shown that the transferase produced by the A^2 gene differs from that produced by the A^1 in the way that it is less efficient in converting H- chains to A; however, the terminal sugar of A^2 is the same as the terminal sugar of A^1 (Bird, 1952).

The human genome encodes two α1,2-fucosyltransferases, FUT1(H) and FUT2 (Se), respectively encoded by *H* and the *Secretor* genes **(Table 1)**.The *Secretor* gene (*Se*) controls the individual's ability to secrete soluble A, B and H antigens into body fluids and secretions (Zmijewski *et al.*, 1968).

The Lewis antigen system is intimately associated with the secretor system and ABO blood group system biochemically and their constitutive structures correspond to related α1-3(4)-fucosylated glycans built up from type 1 and type 2 precursor chains (Clausen and Hakomori, 1989) **(Figure 4)**.

The H-type 1 and H-type 2 antigens are formed by the substitution of the terminal Gal by the α1,2-fucose residue on a type 1 or type 2 chains, respectively. These structures can be further fucosylated by α1,4/3 fucosyltransferases that add a fucose in a α1,4-linkage to the type 1 GlcNAc, originating Lewis b (Le^b) antigen, or in a α1,3-linkage to type 2 structures producing the Lewis Y (Le^y) antigen. Alternatively, the type 1 and type 2 chain backbones may be first modified by α1,4/3 fucosyltransferases producing Lewis a (Le^a) and Lewis x (Le^x) antigens, respectively. Otherwise, the terminal Gal residue of type 1 and type 2 chains can be first modified by sialyltransferases by addition of an α2,3 sialic acid residue, followed by fucosylation of the GlcNAc in α1,4 or α1,3 linkage originating the sialyl-Lewis a (sialyl-Le^a) or sialyl-Lewis x (sialyl-Le^x) structures, respectively.

The transfer of fucose and sialic acid residues to the terminal structures on glycoconjugates are catalyzed by enzymes with different acceptors specificity **(Figure 5)**. With regard to the α1,2 fucosyltyransferase activity, FUT2 acts mostly on type 1 chains and the FUT1 preferentially adds fucose to type 2 chains although it can be acceptor as well of type 1-chains (Holgersson and *Löfling et al.*, 2006; Mollicone *et al.*, 1985). Modification with α2,3-linked sialic acid is mediated by ST3Gal III and ST3Gal IV in both type 1 and type 2 chains leading to sialyl-Le^a and sialyl-Le^x, respectively, and by ST3Gal VI exclusively in type 2 chains, originating sialyl-Le^x. Concerning the α1,4 fucosyltyransferase activity, both FUT3 and FUT5 can add α1,4-linked fucose to type 1 chains resulting in Le^a, Le^b and sialyl-Le^a synthesis (Carvalho *et al.*, 2010; de Vries *et al.*, 2001; Ikehara *et al.*, 1998). The transfer of α1,3- linked fucose to type 2 chains can be mediated by several fucosyltransferases depending on the acceptor backbone, FUT3, FUT4 and FUT9 can participate in Le^y synthesis, whereas FUT3, FUT4, FUT5, FUT6 and FUT9 can add fucose to type 2 chains forming Le^x antigen (de Vries *et al.*, 2001). With respect to the sialylated type 2 chains, the addition of α1,4 fucose is mediated by FUT3, FUT5, FUT6 and FUT7, resulting in sialyl-Le^x expression both on glycolipids and glycoproteins (de Vries *et al.*, 2001). Modification of Lewis antigens can further occur by addition of sulfate groups.

The fucosylated H-type 1 and Le^b antigens are naturally expressed on the gastric mucosa of secretor and Lewis-positive individuals (Magalhaes and Reis, 2010). The secretor status depends on the presence of an active FUT2 enzyme. Inactivating mutations in the secretor gene affect 20% of the human population and have been associated with reduced susceptibility to *H. pylori* infection (Azevedo *et al.*, 2008; Ikehara *et al.*, 2001).

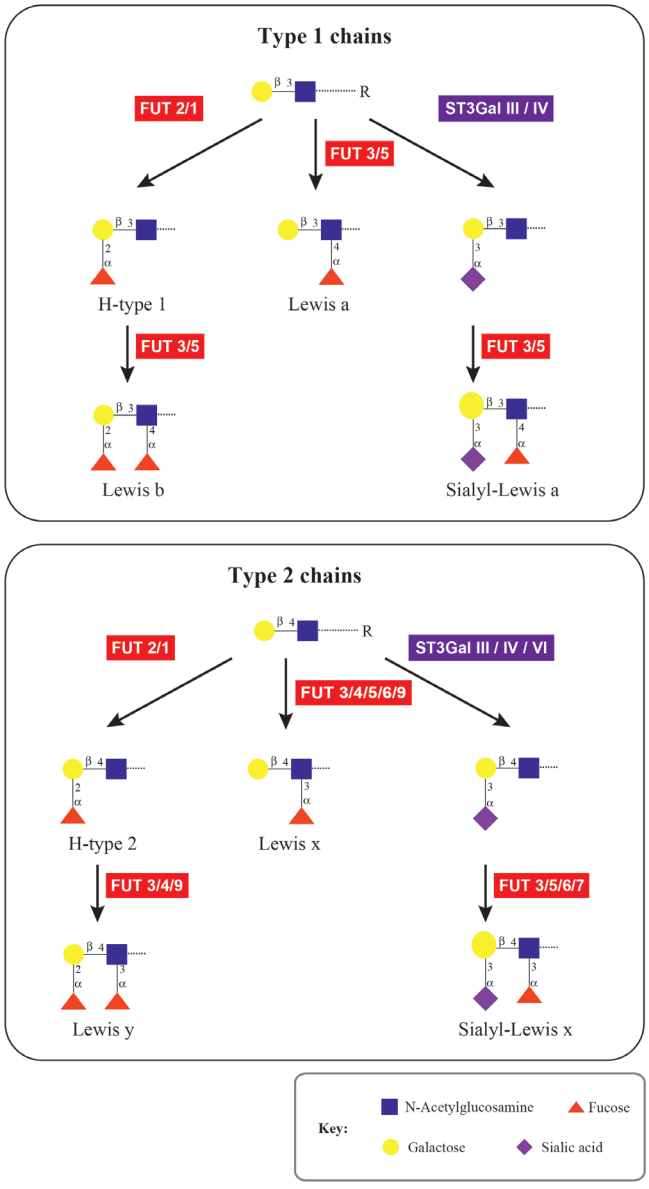


Figure 5 – Schematic representation of type 1 and type 2 Lewis antigens biosynthesis pathways.

Previous studies demonstrated that, in normal human gastric mucosa, type 1 antigens Le^a and Le^b are mainly expressed in the surface of the gastric epithelium. On the contrary, type 2 antigens are found deeper in the gastric glands (Lopez-Ferrer *et al.*, 2000).

Canine blood groups and canine secretory alloantigen alloantibody system (CSA)

The first studies on canine blood groups date from 1910 by Von Dungren (as cited by (Swisher and Young, 1961) and since that, more than 20 different blood groups have been reported in the dog. As dogs do not develop clinically significant naturally occurring alloantibodies most of this filed research have been performed through laboratorial induction of antibodies by mismatched transfusion (Hohenhaus, 2004).

Canine blood groups are currently denominated by the acronym DEA (dog erythrocyte antigen) followed by the numerical designation of the blood group. Seven different groups have received international standardization however, typing sera is only available for five of them (DEA1, DEA3, DE4, DEA5, DEA7/Tr). The DEA7 is also known as Tr which is a soluble antigen that is not produced by the RBC but is absorbed at the cell membrane as similar to the human Lewis antigen (Bull *et al.*, 1975).

The biochemical structure of specific blood group antigens has not been identified but it was determined that the sphingoglycolipids present in the canine erythrocytes membrane contain sialic acid residues (Hohenhaus, 2004).

The described blood groups in dogs have no antigenic or serologic relationship to human blood groups; however, like human intestinal cells, canine intestinal cell fucolipids have human blood group activity. Canine intestinal fucolipids with blood group H activity have an oligosaccharide portion (fucose) identical to that from human erythrocytes (Smith *et al.*, 1975). Blood group A fucolipids are found in canine intestinal glycolipid profiles. Qualitative analysis of sugar composition showed canine intestinal fucolipids with glucose, galactose, glucosamine, galactosamine, and fucose with blood group A activity (Smith *et al.*, 1975).

Antisera against Tr (DEA 7) cross reacts with human group A cells; consequently, the Tr antigen appears to have similarity with the A antigen of human A^1 red blood cells (Bowdler *et al.*, 1971). Additionally, the saliva of Tr positive dogs contains anti-A and anti-H activity (Bowdler *et al.*, 1973). Despite the presence of anti-H activity in canine saliva, no H substance has been identified on canine erythrocytes, but it has been identified in canine intestine cells (Smith *et al.*, 1975).

The canine secretory alloantigen alloantibody system (CSA) is closely related to the human ABH-Le system being considered equivalent to the major human ABO exocrine glycophenotype. The CSA was first described in 1966 by Zweibaum and colleagues (Zweibaum *et al.*, 1966). The genetic polymorphisms of the CSA were later analysed (Zweibaum *et al.*, 1974) and the structure of canine polymorphic antigens was further characterized in four phenotypes: A, X, Y, and AY whose structures and chemical linkages were proposed based on inhibition tests observed with oligosaccharides (Oriol *et al.*, 1975) (**Figure 6**). The same authors reported the presence of only type 2 chains in dog intestinal secretions. No further information is available about the glycosylation profile of the canine gastric mucosa

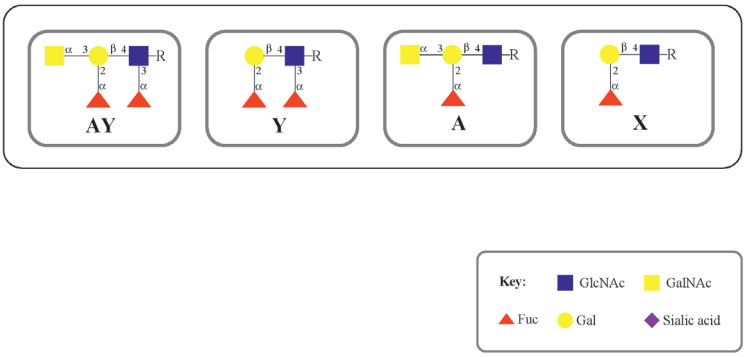


Figure 6 - Schematic representation of canine secretory alloantiagens (CSA) polymorphisms. (Adapted from Oriol *et al.*, 1975)

Lectins

Lectins are natural carbohydrate-binding proteins often found in extracts of seeds from plants, which react specifically with certain carbohydrate antigens. Two common and relevant forms are the *Ulex europaeus* or lectin H, which agglutinates cells that have H antigen; and another that has been used in the present study is *Dolichos biflorus*, lectin DBA or A^1, which agglutinates cells with A^1 (Bird, 1952).

Mucins

Mucins are a family of high molecular weight, heavily O-glycosylated proteins expressed by several epithelial tissues that can be produced either as membrane bound or as secreted proteins (Kufe, 2009).

In the human gastric mucosa, the mucus gel that covers the mucosal surface represents the interface between the epithelial cell layers and the exterior environment and is composed of a high carbohydrate content of mucins (Linden *et al.*, 2008b). The mucus

gel has a thickness of approximately 300 μm and can be divided in two layers: one firmly attached to the mucosa whose thickness increases, from the body to the antrum regions, with values ranging from 80 to 154 μm (Linden *et al.*, 2008b; Moore *et al.*, 2011; Phillipson *et al.*, 2008) and other layer more loosely adherent.

The mucus gels acts as a physical barrier that lubricates and defends the epithelial cells from external aggressions displaying important protective properties. Furthermore, it allows the establishment of a pH gradient with the bicarbonate ions secreted by the surface epithelial cells counteracting the diffusion of luminal acid, leading nearly to a neutral pH at the cell surface while the gastric lumen displays acidic properties (Bhaskar *et al.*, 1992).

The human mucin (MUC) family includes 21 members (MUC1 to MUC21) (Kufe, 2009). In a healthy gastric mucosa, the mucins produced are MUC1, MUC5AC and MUC6. The membrane-associated MUC1 is expressed in foveolar cells and, to a lesser extent, in mucous glands. The secreted MUC5AC mucin is restricted to the foveolar epithelium and is a major constituent of the surface mucous gel layer, whereas the expression of the secreted MUC6 is limited to the glands (Reis *et al.*, 2000; Reis *et al.*, 1997; Reis *et al.*, 1998; Teixeira *et al.*, 2002). This mucin distribution determines the gastric glycosylation pattern since expression of MUC5AC is accompanied by similar distribution of fucosyltransferases leading to co-expression of type 1 Lewis a (Le^a) and Lewis b (Le^b) blood group antigens, while MUC6 expression is associated with the type 2 Lewis x (Le^x) and Lewis y (Le^y) antigens (Magalhaes and Reis, 2010).

Mucins are frequently regarded as preferential binding sites for infectious agents, mainly because of their high glycans content (Linden *et al.*, 2008b). This may be beneficial for the host as they act as releasable decoys in which the pathogens are trapped and them removed and eliminated (Linden *et al.*, 2008a; Moore *et al.*, 2011). In contrast, the binding to specific glycans in mucins may represent a threat to the host since it allows the pathogens to establish a more intimate contact with epithelial cells favoring the colonization.

Virtually all interactions between microbial pathogens and their hosts are mediated by cell surface expressed glycans. Therefore it is expected that both the host and the pathogen glycans landscape will define the pathogen tropism for a particular organ, tissue or specific cell. The glycan-receptor interactions may be essential for the initial colonization of host epithelial cells and establishment of infection, but can also favour microbe recognition by the host immune cells, stimulating an immune response.

An example of a bacterium that exploits the glycans receptors expressed by the host cells to survive and successfully colonize a tough niche is the human gastric pathogen *Helicobacter pylori* (Magalhães and Reis, 2010),.

- Gastric *Helicobacter spp.* in humans and dogs

The first description of a gastric member of the *Helicobacter* genus was reported by Marshall and Warren in 1984 (Marshall and Warren, 1984). The discovery of the association between the presence of these bacteria in the human stomach and ulcer diseases awarded Marshall and Warren the Nobel Prize of Medicine and Physiology in 2005. The causative agent, ***Helicobacter pylori*** *(H. pylori)*, has also been associated with gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa associated lymphoid tissue (MALT) lymphoma (Parsonnet *et al.*, 1991; Stolte and Eidt, 1993).

H. pylori is a microaerophilic, Gram-negative, spiral-shaped bacterium 0.5-1.0 µm wide and 2.4-4.0 µm long, with 5 to 7 unipolar sheathed flagella that colonizes the human stomach of one half of the human population worldwide (Forman *et al.*, 1993).

In developing countries and low socio-economic classes the bacterium is usually acquired in infancy or early childhood and the infection prevalence is high with more than 80% of the population being infected (Holcombe *et al.*, 1992; Segal *et al.*, 2001). On the other hand, in developed countries, the prevalence of *H. pylori* usually remains under 40% and is considerably lower in children and adolescents than in adults and elderly people (Pounder *et al.*, 1995; Kusters *et al.*, 2006). In most individuals, *H. pylori* infection is asymptomatic. About 25% to 30% of infected individuals will, however, one day experience the disease.

In addition to *H. pylori*, another **non-*Helicobacter pylori* *Helicobacter*** (NHPH) organism was found in the stomach of patients suffering from dyspepsia (McNulty *et al.*, 1989). This tightly coiled organism, with 10 to 20 bipolar sheathed flagella, was clearly distinguishable from *H. pylori*. This bacterium was first referred to as "*Gastrospirillum hominis*". However, following 16S rRNA sequencing, it was designated to the *Helicobacter* genus and the name "*H. heilmannii*" was proposed (Heilmann and Borchard, 1991; Solnick *et al.*, 1993).

NHPHs generally referred to as "*H. heilmannii*" were further subdivided in two taxa, types 1 and 2 (Haesebrouck *et al.*, 2011). Microorganisms referred to as *H. heilmannii* type 1 are identical to *H. suis*, a species colonizing the stomachs of pigs (De Groote *et al.*, 1999). The former *H. heilmannii* type 2 represents a group of species known to colonize the gastric mucosa of dogs and cats, which includes *H. felis, H. bizzozeronii, H. salomonis, H. cynogastricus, H. baculiformis* and a bacterium which was given the provisional name of ''*Candidatus H. heilmannii*'' in 2004 because, at that time, it could not be cultured *in vitro* (O'Rourke *et al.*, 2004). In 2012, a description of *H. heilmannii* as a novel species was achieved (Smet *et al.*, 2012). The name ***H. heilmannii sensu stricto*** (s.s.) was proposed

to refer to the novel *Helicobacter* species and the term *H. heilmannii sensu lato* (s.l.) to refer to the whole group NHPH (Haesebrouck *et al.*, 2011).

Since the description of *H. pylori*, the number of species belonging to the genus *Helicobacter* rapidly expanded and currently it is composed of at least 32 species with validly published names (Haesebrouck *et al.*, 2009). A large number of NHPH species have been recognized in humans and in a wide variety of animals, including dogs.

H. felis was first cultured by Lee and colleagues (Lee *et al.*, 1988) from the stomach of a cat and later, the group of Paster also isolated this organism from canine gastric mucosa (Paster *et al.*, 1991). This organism is tightly coiled and possesses 14 to 20 bipolar sheathed flagella. The presence of periplasmic fibrils that encase the bacterium was thought to be a unique feature of this gastric *Helicobacter* species until the isolation of ***H. cynogastricus*** from the canine mucosa (Van den Bulck *et al.*, 2006).

Later, another spiral organism morphologically distinct from *H. felis* was isolated from the canine mucosa (Hanninen *et al.*, 1996). This organism lacked periplasmic fibrils around its thin body and had 10 to 20 bipolar flagella. It was named as ***H. bizzozeronii*** after Guilio Bizzozero confirmed by DNA-DNA hybridisation that it was a new *Helicobacter* species. Then, ***H. salomonis*** was isolated from dogs and it was named in honour of Hugo Salomon (Jalava *et al.*, 1997). It has 10 to 23 bipolar sheathed flagella and no periplasmic fibrils.

Several genome sequences of *H. pylori* are available and in the last few years, the genomes of other dog-related gastric *Helicobacter spp*. were also published. The genomes of the type strain of *H. felis* isolated from a cat (Arnold *et al.*, 2011); of *H. bizzozeronnii* strain CIII-1 obtained from the human stomach (Schott *et al.*, 2011b) and of *H. heilmannii s.s.* isolated from the gastric mucosa of a kitten with severe gastritis (Smet *et al.*, 2013) were reported. Additionally, the *H. salomonis* genome has been recently completed, but results have not yet been published.

The comparative genome analysis between *H. pylori* and these dog-related NHPH have provided new insights into the comprehension of the biology of these particular gastric organisms belonging to the *Helicobacter* genus (Arnold *et al.*, 2011; Eppinger *et al.*, 2006; O'Toole *et al.*, 2010; Schott *et al.*, 2011b; Smet *et al.*, 2013).

- Gastric *Helicobacter spp.* related disease in humans and dogs

Helicobacter pylori is the main cause of gastric inflammation in virtually all infected subjects (Farinha and Gascoine, 2005). Most of the infected individuals show few or none symptoms, and only a small percentage will develop severe gastric disease (Amieva and El-Omar, 2008).

The first and acute phase of *H. pylori* human infection consists of polymorphonuclear cells infiltration into the gastric epithelium and lamina propria, accompanied by oedema. This step is very brief and is gradually replaced by a chronic active inflammatory response characterized by progressive mononuclear cells infiltration, mucosa-associated lymphoid tissue, as well as damage to the epithelial cells. The pattern and distribution of chronic gastric inflammation is associated with the type of lesions observed: individuals with body-predominant gastritis and normal or reduced acid production are more likely to develop gastric ulcers, gastric atrophy, gastric intestinal metaplasia and ultimately, gastric carcinoma while individuals with antral-predominant gastritis, which is the most common form, show increase acid production and increased risk to develop duodenal ulcers (Kusters *et al.*, 2006; Lochhead and El-Omar, 2007). Additionally, *H. pylori* has been responsible for 70-85% of gastric ulcers and 90-95% of duodenal ulcers (Kusters *et al.*, 2006).

The next phase of human *H. pylori* infection is dominated by alterations of the epithelial cell cycle, especially increased rates of apoptosis and cell proliferation. These changes may be responsible for the multifocal atrophy that characterizes the type of gastritis associated with an increased risk of cancer. In this more advanced phase nuclear and architectural abnormalities become notice, which may represent progressive mutational events as expected in classical molecular models of carcinogenesis (Correa, 2004).

Although only a small proportion of patients with *H. pylori* will eventually develop malignant disease, the widespread high prevalence of this bacterium explains that gastric cancer remains the fifth most common cancer worldwide (Globocan, 2012). Thus, based on epidemiological evidences, in 1994 the International Agency for Research on Cancer classified *H. pylori* as a class I carcinogen (IARC, 1994). In fact, *H. pylori* infection has a pivotal role in the development of two different gastric malignancies: gastric adenocarcinoma and gastric MALT lymphoma (Farinha and Gascoyne, 2005; Parsonnet, 1994; Parsonnet *et al.*, 1991; Stolte *et al.*, 2002)

Gastric adenocarcinoma is subclassified into intestinal and diffuse types (Lauren, 1965). The sequence of pathological changes leading to the development of an intestinal-type start with gastritis, followed by gastric atrophy and progressing to intestinal metaplasia,

dysplasia and ultimately, carcinoma (Correa, 2004). Most of the individuals affected by this particular subtype are middle aged or older. The diffuse-type of gastric cancer tends to occur in younger individuals and has a stronger genetic component (Carneiro, 2012).

The association between MALT-lymphoma and *H. pylori* was first reported in 1991 (Wotherspoon *et al.*, 1991). *H. pylori* is responsible for 92 to 98% of gastric MALT-lymphomas (Mbulaiteye *et al.*, 2009). Based on recent knowledge about the existence of genetic abnormalities in this kind of lesion, a model of multistep pathogenesis was proposed: on the background of the chronic inflammation caused by *H. pylori* not only reactive B-cells are stimulated but also activated neutrophils, which can lead to production of oxygen species. As a result, this genotoxins provoke DNA damages that are responsible for mutations and transformations of genetic material and consequently, genetic instability (Farinha and Gascoyne, 2005; Witkowska and Smolewski, 2013).

The diagnosis of *H. pylori* infection can be done by invasive methods such as endoscopy followed by biopsy (in order to perform histology or culture) or non-invasive tests like urea breath test and serological tests (Correa, 2004; Kusters *et al.*, 2006; Suerbaum and Michetti, 2002). On histology, *H. pylori* are identified on the basis of their typical localization and their characteristic slightly curve-shaped morphology.

Although less often, gastric NHPH are also able to cause disease in humans. In the human stomach, NHPH infections may be accompanied by acute gastritis (Lavelle *et al.*, 1994; Yoshimura *et al.*, 2002), active chronic gastritis (Haesebrouck *et al.*, 2009), erosions mainly located in the antrum (Boyanova *et al.*, 2003; Debongnie *et al.*, 1998; Dieterich *et al.*, 1998; Seo *et al.*, 2003) and duodenal ulcers (Borody *et al.*, 1991; Goddard *et al.*, 1997; Iwanczak *et al.*, 2012; Jhala *et al.*, 1999). Glandular atrophy and intestinal metaplasia of the fundic gastric mucosa were reported. However, all these lesions seem to be less common and less severe than those associated with *H. pylori* (Yoshimura *et al.*, 2002).

Human NHPH infections have also been associated with low-grade MALT lymphoma of the stomach, and the risk of developing this disease is higher with NHPH than with *H. pylori* (Morgner *et al.*, 1995; Morgner *et al.*, 2000a; Morgner *et al.*, 2000b). They have been reported to resolve after clearance of the NHPH, thus emphasizing a causal relationship (Morgner *et al.*, 2000b; Regimbeau *et al.*, 1998; Thomas-Marques *et al.*, 2005).

Some human patients are asymptomatic (Mazzucchelli *et al.*, 1993) while others refer atypical complaints such as acute or chronic epigastric pain and nausea, hematemesis, recurrent dyspepsia, irregular defecation frequency and consistency, vomiting, heartburn, dysphagia and loss of appetite (Dieterich *et al.*, 1998; Goddard *et al.*, 1997; Heilmann and

Borchard, 1991; Iwanczak *et al.*, 2012; Kaklikkaya *et al.*, 2002; Mention *et al.*, 1999; Oliva *et al.*, 1993; Roehrl *et al.*, 2012; Seo *et al.*, 2003; Sykora *et al.*, 2003; van Loon *et al.*, 2003; Wuppenhorst *et al.*, 2013; Yang *et al.*, 1998).

Histologically, the presence of NHPH in human gastric mucosa is mainly characterized by lymphocytic infiltration into gastric foveolae, sometimes accompanied by plasma cells. Lymphoid aggregates may be present and in some cases, superficial mucus was depleted (Flejou *et al.*, 1990; Ierardi *et al.*, 2001; Joosten *et al.*, 2013; Kaklikkaya *et al.*, 2002; Oliva *et al.*, 1993).

Human infections with NHPH most likely originate from animals. Indeed living in close proximity to animals has been identified as a risk factor (Lavelle *et al.*, 1994; Meining *et al.*, 1998; Thomson *et al.*, 1994). The intensity of this contact also seems to be important since a higher incidence of these infections was noted in farmers, staff of slaughterhouses, veterinarians, pet owners and children having intense contact with pets (De Bock *et al.*, 2007; Joosten *et al.*, 2013). In agreement, the prevalence of porcine, canine and feline helicobacters in human gastric biopsies was documented and *H. suis*, *H. felis*, *H. bizzozeronii* and *H. salomonis* were present in 39.6%, 14.6%, 4% and 21%, respectively (Van den Bulck *et al.*, 2005). Recently, the relationship between pet ownership or frequent exposure to dogs and infection with different gastric *Helicobacter* species was assessed (Chung *et al.*, 2014). A significant correlation was found between human and canine infection for *H. felis* and to a lesser extent for *H. bizzozeronnii*. On the other hand, no clear association with animal contact was found in a study who reported a 0.2% of incidence of gastric NHPH infection in dyspeptic Polish children (4–18 years of age) (Iwanczak *et al.*, 2012).

Although *H. pylori* and these dog-related NHPH colonise similar niches, an important difference between both is the later capability to move from a dog to a human (Haesebrouck *et al.*, 2009). Thus, when transferring from dogs to humans, NHPH necessarily undergo intensive changes to adapt to a new host. This NHPH ability for ongoing host-adaptation is probably related to their high level of genome plasticity (Schott *et al.*, 2011a).

In summary, NHPH are of zoonotic significance and the dog remains a natural reservoir for many species (Haesebrouck *et al.*, 2009). Further information concerning the presence of these bacteria in the canine stomach and their pathogenic implications are described ahead in this chapter (paper I).

- Pathogenesis of *Helicobacter spp.* infections

Most of the research concerning the pathogenesis of *Helicobacter* infections is focus on *H. pylori*.

The success of long-term gastric mucosa colonization by *H. pylori* is dependent on several bacterial factors and of specific properties of this bacterium (Amieva and El-Omar, 2008): 1) its urease activity enables it to survive in the extreme acidic conditions of the human stomach (Eaton *et al.*, 1991); 2) its high motility allows it to move easily in the viscous mucus of the stomach (Eaton *et al.*, 1992); 3) its capability to adhere to the gastric epithelium due to the presence of several adhesins (Boren *et al.*, 1993; Hessey *et al.*, 1990 and 4) its ability to escape to the immune host response.

The clinical outcome of *H. pylori* infection is diverse and dependent on the *H. pylori* strain virulence characteristics, host genetic susceptibility, environmental factors and their interactions. In contrast, very little is known about the specific direct and indirect pathogenic effects of other gastric NHPH and about differences in pathogenicity between different strains within the same species of NHPH (Haesebrouck *et al.*, 2009).

Acid Acclimation

An important mechanism of survival that is required for the colonization of the stomach and that is share by all gastric *Helicobacter* species is the production of urease (Haesebrouck *et al.*, 2009). This enzyme, which hydrolyzes urea to ammonia and carbon dioxide leading to a local pH increase, allows bacteria to survive and to multiply and consists of two subunits: UreA and UreB, which are encoded by two genes *ureA* and *ureB* (Hu and Mobley, 1990). Urease enzyme activity is tightly controlled by a pH-gated urea channel (ureI), which is open at low pH and closed at neutral pH conditions, allowing the bacterium a precise level of control over its pH environment. Urease is mainly localised in the cytoplasm but becomes associated with the surface of the viable bacteria after autolysis of surrounding *Helicobacters* (Krishnamurthy *et al.*, 1998; Marcus and Scott, 2001; Phadnis *et al.*, 1996). However, the contribution to acid resistance is solely the result of the cytoplasmic urease (Scott *et al.*, 1998).

The two subunits UreA and UreB of *H. felis* urease are considered homologous but not identical to those of *H. pylori* (Ferrero and Labigne, 1993; Gootz *et al.*, 1994). A second urease system was also identified in this species - the UreA2B2 - but its function and regulation are unknown (Pot *et al.*, 2007). High homology between the urease gene cluster of *H. bizzozeronnii* and those of *H. felis* and *H. pylori* is also documented (Zhu *et al.*, 2002).

However, *H. bizzozeronnii* lacks the additional urease gene (Schott *et al.*, 2011a).

The presence of urease in the stomach is the basis of the most widely used noninvasive test for human *H. pylori* diagnosis, the urea breath test. A recent study concluded that this method is also quite useful for the detection of gastric *Helicobacter spp.* infection in dogs (Kubota *et al.*, 2013).

Motility and Chemotaxis

Motility is fundamental for colonization of the mucosa by gastric *Helicobacter* species (Fischer *et al.*, 2009).

Gastric helicobacters possess monopolar (*H. pylori*) and bipolar (*H. felis, H. bizzozeronnii, H. salomonis, H. heilmannii s.s., H. cynogastricus*) bundles of 2 to 23 flagella. The flagella is composed of a body, hook and flagellar filament. The complex flagellar filament is covered by a sheath and is composed of two flagellin subunits: the more abundant is the FlaA and the minor is the FlaB. It was verified that both subunits are necessary for full motility *in vitro* (Josenhans *et al.*, 1995) and *in vivo* (Eaton *et al.*, 1996). Additionally, the sheath is suspected to play a role in acid protection, masking of antigens and possible adhesion (Jones *et al.*, 1997).

Josenhans *et al.* (1999) cloned *H. felis flaA* and *flaB* genes and mutants were constructed. These mutants were described as poorly motile *in vitro* and the loss of the ability to colonize the mouse stomach of the *flaA* mutant was reported. Thus, it was assumed that any impairment in motility would have consequences for gastric colonisation by *H. felis* (Josenhans *et al.*, 1999).

The basal body of the flagella is embedded in the bacterial cell wall and contains the proteins required for rotation and chemotaxis. Bacteria use chemotaxis to migrate towards environments that are better for growth. Chemoreceptors detect changes in attractant levels and signal through two-component systems to control the swimming direction (Porter *et al.*, 2011). Chemotactic pathways that are governed by two-component regulatory systems, histidine kinase proteins and regulatory proteins, modulate the direction and speed of motility using responses of specific signals detected by receptors formed by methyl-accepting chemotaxis proteins (MCPs). The repertoire of the chemotactic signal pathways of a microorganism is associated to the environments it can transfer, the hosts it colonises and the diseases it can cause (Szurmant and Ordal, 2004).

Schott *et al.* (2011) when analysing the *H. bizzozeronni* genome found that this

particular species harbours five times more MCPs than *H. pylori* (Schott *et al.*, 2011a). The abundance of predicted MCPs in *H. bizzozeronnii* and *H. felis* genome indicates that both bacteria possess an elaborate sensing capability that may allow their survival in different ecological niches and probably supports their capability to transfer between different hosts (Arnold *et al.*, 2011; Schott *et al.*, 2011a).

Adhesion

It is well known that *H. pylori* adhesion to the gastric epithelial cells constitute a crucial step for gastric mucosa colonization and establishment of a successful infection, because it provides protection from clearance mechanisms such as liquid flow, peristaltic movements or renewal of the mucous layer (Magalhaes *et al.*, 2010). Nevertheless this attachment may also represent some risks to the bacteria since it increases proximity with the host immune response effector molecules and bacteria may be shed to the lumen by the frequent epithelium cells turn-over.

Although most *H. pylori* bacteria are found free-swimming in the mucus layer overlying the gastric epithelium, a minor fraction of bacteria adheres to the gastric surface epithelial cells (Schreiber *et al.*, 2004). Also *H. felis* is mainly found in the mucus layer and adherence to epithelial cells is believed to be rare (Schreiber *et al.*, 1999). Nevertheless, some authors have reported the ability of this bacterium to attach to host cells (Haziroglu *et al.*, 1995; Lecoindre *et al.*, 2000; Peyrol *et al.*, 1998). Furthermore, Haziroglu and colleagues described adhesion of *H. felis* to the surface of canine parietal cells (Haziroglu *et al.*, 1995).

The analysis of the complete genome sequence of *H. pylori* strains revealed that near 4% of the bacteria coding potential, encodes a diverse repertoire of outer membrane proteins (OMPs) (Odenbreit, 2005; Tomb *et al.*, 1997). This large group of OMPs contains some adhesins that mediate bacterial binding to the host cell membrane, such as: blood-group antigen-binding adhesin (BabA), sialic acid binding-adhesin (SabA), adherence-associated lipoprotein A and B (Alp A/B); outer inflammatory protein A (OipA); and other factors (e.g. lipopolysaccharide (LPS) and flagellin, which are able to trigger inflammatory responses in host tissues. However, only two of those proteins have been well characterized regarding its host receptors and role on bacterial adhesion (Ilver *et al.*, 1998; Mahdavi *et al.*, 2002).

The **blood group antigen-binding adhesin (BabA)**, binds to fucosylated blood group antigens H-type 1 and Le^b (Boren *et al.*, 1993; Ilver *et al.*, 1998) antigens are expressed in the gastric mucosa epithelium of healthy secretor individuals. Thus, infection by BabA-positive strains is dependent on the individuals' secretor status (Azevedo *et al.*, 2008). The H-type 1 and Le^b glycan structures are also extensively present in the MUC5AC mucin,

mediating the binding of *H. pylori* strains expressing the BabA adhesin to the gastric mucus layer (Linden *et al.*, 2002; Van de Bovenkamp *et al.*, 2003). The frequency of *H. pylori* strains expressing the BabA adhesin ranges from 35% to 70% in European populations while in Japan, Korea, Taiwan, Columbia and United States this percentage is higher than 60% (Gerhard *et al.*, 1999; Ilver *et al.*, 1998; Lee *et al.*, 2006; Olfat *et al.*, 2005; Prinz *et al.*, 2001; Sheu *et al.*, 2003; Yu *et al.*, 2002; Zambon *et al.*, 2003).

The persistent *H. pylori* colonization in the human stomach promotes gastric mucosa inflammation that is accompanied by a replacement of the neutral glycans structures normally expressed by the charged sialylated antigens, sialyl-Le^a and sialyl-Le^x (Mahdavi *et al.*, 2002; Ota *et al.*, 1998). These sialylated structures are the receptors for the **sialic acid-binding adhesion (SabA)** that mediates bacterial binding to inflamed gastric mucosa (Mahdavi *et al.*, 2002). Previous studies on European populations suggest that almost 40% of the clinical isolates express SabA (Aspholm *et al.*, 2006; Mahdavi *et al.*, 2002; Odenbreit *et al.*, 2009). A study addressing clinical isolates from Taiwan suggests that SabA interaction with the host gastric sialyl-Le^x antigen is particularly relevant in patients with lack or weak Le^b expression, enhancing *H. pylori* colonization (Sheu *et al.*, 2006).

The rapid modulation of BabA and SabA adhesins expression is an important ability of *H. pylori* which allows the progressive adaptation of the bacteria to the constant changes of the gastric mucosa and is considered an essential feature for achievement of long term colonization.

Although BabA and SabA are so far the *H. pylori* adhesins for which the receptors are well known, not every strain expresses these functional adhesins, suggesting that other bacterial proteins are certainly involved in *H. pylori* adhesion to the gastric cells.

Two homologous OMPs, the adherence-associated lipoprotein A and B (AlpA and AlpB) have been demonstrated to be relevant for *H. pylori* adhesion to a gastric cell line and human gastric biopsies (Odenbreit *et al.*, 1996) (Odenbreit *et al.*, 2002; Odenbreit *et al.*, 1996; Odenbreit *et al.*, 1999). Additionally, a study has proposed that *H. pylori* lipopolysaccharide (LPS) side chains expressing Le^x, could also mediate adhesion to gastric mucosa (Edwards *et al.*, 2000). However, this evidence was not supported by latter experiments which indicated that LPS-Le^x has a limited role in promoting binding to gastric tissues (Mahdavi *et al.*, 2003; Odenbreit *et al.*, 2002).

Recently, a new bacterial adhesin of *H. pylori*, termed lacdiNAc-binding adhesin (LabA), has been identified (Rossez *et al.*, 2014).

Little is known about the adhesins recruited by NHPH. Recently, it has been shown that NHPH OMPs genes share sequence homology with *H. pylori*. However, among the

dog-related helicobacters, none present in their genomes orthologues of *H. pylori* adhesins. The genome of *H. bizzozeronii, H. felis, H. salomonis* and *H. heilmannii s.s.* encodes several OMPs but lacks the Bab and Sab adhesins which are important in *H. pylori* (Arnold *et al.*, 2011; Schott *et al.*, 2011a; Smet *et al.*, 2013). Furthermore, the *alpA* and *alpB* genes are not present in *H. felis, H. heilmannii s.s.* and *H. bizzozeronnii* (Odenbreit *et al.*, 1999; Schott *et al.*, 2011b).

Other putative adhesins of dog-associated NHPH have not yet been described.

Virulence

The difference of pathogenicity of *H. pylori* strains isolated from patients could be explained by the presence of specific virulence factors (Atherton, 2006):

cagPAI – cytotoxin-associated gene pathogenicity island

The *cagPAI* is a multigenic region encoding, among others, the CagA protein, a cytotoxin-associated gene that by itself could indicate the presence or absence of the cytotoxin-associated gene pathogenicity island (*cagPAI*). The *cagPAI* genes encode components of the type IV secretion system that allows bacterial proteins to enter into host epithelial cells (Censini *et al.*, 1996). CagA is one of the bacterial proteins that are translocated and once in the host cell cytoplasm it is phosphorylated on tyrosine residues within the Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs. This phosphorylation of CagA is important for the cytoskeleton rearrangement. CagA is associated as well with disruption of tight and adherent junctions. Furthermore CagA is also required for released of IL-8 that promotes the recruitment of inflammatory cells in the gastric mucosa (reviewed in Atherton, 2006). *H. pylori* strains are classified as cagPAI positive, virulent strains whose infected individuals have increased risk of gastric cancer development or cagPAI negative that are less virulent strains (Atherton, 2006; Parsonnet *et al.*, 1997).

vacA – vacuolating cytotoxin

The *vacA* is the gene that encodes a protein secreted by *H. pylori* that induces cells vacuolisation membrane channel formation, disruption of endosomal/liposomal function, epithelial-cell apoptosis and immunomodulation. It was identified in all strains and comprises two variable regions: the s region (secretion-signal region) located at the 5' end of the gene and existing in two allelic forms, s1 (subtype a or b) or s2; and the m region (mid-region), with m1 or m2 alleles. These allelic regions have different capacity for the production of this cytotoxin. Combination of *vacA* gene with s1/m1 alleles are associated with increased inflammation and high risk of development of the disease due to its high vacuolating activity.

On the other hand, vacA s2/m2 does not present cytotoxin activity and is not associated with disease. There are also studies that associate the presence of *CagA* with *vacA* s1 (Atherton *et al.*, 1995; Cover and Blanke, 2005).

NapA – *neutrophil-activating protein*

The neutrophil-activating protein is encoded by the *napA* gene which is homologous to other bacterioferritins. Contrary to ferritins that store iron and bind DNA, the *H. pylori* NapA crosses both the epithelium and endothelium where it increases the adhesion of neutrophils to endothelial cells and stimulates both neutrophils and monocytes to produce interleukin (IL)-12 and IL-23 (Amedei *et al.*, 2006; de Bernard and D'Elios, 2010). These cytokines induce T cells to secrete interferon- ɣ and mediate the shift to a Th1 response (Amedei *et al.*, 2006). Min Long and colleagues found significant correlation between serum NapA antibodies level in *H. pylori*-infected patients and healthy controls (Long *et al.*, 2009). Additionally, seropositivity for *H. pylori*-NapA specific antibodies in gastric cancer patients was higher than that in chronic gastritis patients and healthy controls (Long *et al.*, 2009; Luo *et al.*, 2007).

HtrA – *high temperature requirement*

The HtrA is a periplasmic chaperone and serine protease present in *H. pylori* that is highly stable under extreme acidic stress conditions contributing to the establishment of persistent infection *in vivo* (Hoy *et al.*, 2013). This protease is composed by an N-terminal signal peptide, a serine protease domain with a highly conserved catalytic triad, and two PDZ (postsynaptic density protein, *Drosophila* disc large tumour suppressor and zonula occludens-1 protein) domains. Recently, it has been demonstrated that *H. pylori* HtrA is secreted into the extracellular space as an active serine protease (Lower *et al.*, 2008). Here, it cleaves the extracellular domains of the cell adhesion molecule and tumour suppressor E-cadherin, leading to a local disruption of adherence junctions of polarized gastric epithelial cells that allow the bacteria entrance into the intercellular space. This evidence was supported by electron microscopy analyses of biopsy samples from cancer patients in which this bacterium was found in the intercellular space and at the basal lamina (Necchi *et al.*, 2007).

The identification of *H. pylori*-secreted HtrA as an E-cadherin protease revealed how *H. pylori* uses HtrA as a bacterial protease in a coordinated manner with the injected effector protein, cytotoxin-associated gene A. This leads to the destruction of the epithelial barrier function, allowing persistent *H. pylori* colonization, nutrition and pathogenesis (Wessler and Backert, 2008). Furthermore, this finding supports the hypothesis that bacterial HtrA not only indirectly influence microbial pathogenicity through improvement of bacterial fitness under stress conditions, but also exhibits direct effects on infected host cells (Posselt *et al.*, 2013).

Although homologous *cag*PAI and *vac*A genes have not been found in gastric NHPH, vacuolation and necrosis of parietal cells has been observed in the gastric mucosa of dogs naturally infected by *Helicobacter spp*. (Happonen *et al.*, 1996; Hermanns *et al.*, 1995; Yamasaki *et al.*, 1998). It was also shown that *H. felis and H. bizzozeronnii* induce, respectively, extensive and focal apoptosis and necrosis of parietal cells in experimentally infected gerbils (De Bock *et al.*, 2006). Indeed, *H. pylori* and NHPH share virulence factors involved in epithelial cell death such as the ɣ-glutamyl transpeptidase (Haesebrouck *et al.*, 2009). Furthermore, genes enconding homologues of other *H. pylori* virulence factors such as the secreted serine protease HtrA and the imunomodulator NapA are present in *H. felis*, *H. bizzozeronnii* and *H. heilmannii s.s.* genomes.

In *H. bizzozeronnii* a polysaccharide lyase was regarded as a potential new virulence factor (Schott *et al.*, 2011b). Later Kondadi *et al.* (2012), characterised a novel lipopolysaccharide α2,3-sialyltransferase from *H. bizzozeronii* that showed a preference for N-acetyllactosamine as a substrate. The authors showed that the expression of a terminal 3′sialyl-LacNAc on LPS is a phase-variable characteristic of both human- and canine-derived *H. bizzozeronii* strains. Moreover, the authors suggest that this sialylated structure can mimic the surface glycans of the host mammalian cells (Kondadi *et al.*, 2012).

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Paper I

Unravelling canine gastric pathology

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Revision article

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Table of Contents

0. Introduction
1. Acute non-erosive gastritis
2. Gastric erosions and ulceration
3. Chronic gastritis (superficial and transmucosal):
    3.1. Lymphoplasmacytic gastritis
    3.2. Eosinophilic gastritis
    3.3. Granulomatous gastritis
4. Gastric mucosal fibrosis and atrophy
5. *Helicobacter spp*. infection
6. Gastric proliferative diseases
    6.1. Hypertrophic gastritis
7. Gastric polyps
8. Special forms of gastropathy
    8.1. Hypertrophic pyloric gastropathy
    8.2. Uremic gastropathy
9. Gastric metaplastic changes
    9.1. Osseous metaplasia/heterotopic bone formation
    9.2. Glassy cell change (GCs)
    9.3. Pseudopyloric or epithelial mucous metaplasia
    9.4. Intestinal metaplasia (IM)
10. Gastric dysplasia
    10.1. Epithelial dysplasia
    10.2. Globoid dysplasia (GD)
11. Gastric epithelial neoplasia
    11.1. Epidemiological features of canine gastric carcinoma (GC)
    11.2. Classification of gastric carcinoma
        - Early and Advanced Gastric Carcinoma
        - Histological classification of Gastric Carcinomas
    11.3. Application of Molecular Pathology in Gastric Carcinoma
12. Conclusions

Introduction

Canine gastric disease is typically the result of inflammation, ulceration, obstruction or neoplasia. The present manuscript reviews and classifies these disorders. Canine gastritis is categorized and graded subjectively according to the nature of the predominant cellular infiltrate and the presence of architectural abnormalities (Day *et al.*, 2008). In contrast, the term 'gastropathy' is used to refer to a nonspecific injury pattern of the gastric mucosa, with minimal or no inflammatory cell infiltration (Dixon *et al.*, 1996). In man, some lesions of the gastric mucosa develop as a sequence of genotypic and phenotypic alterations, which may lead to neoplasia (Correa, 1992). In veterinary medicine such sequential pathogenesis is not yet defined, although metaplastic and dysplastic changes are also recognized in the canine stomach (Head, 2003). Canine gastric cancer has been well described and is now beginning to be explored at the molecular level. This spectrum of gastric pathology is described herein by systematic review of the classification, pathophysiology and macroscopic and histopathological findings of the most common canine gastric lesions.

1 - Acute non-erosive gastritis

In pathological terms, gastritis is defined as inflammation of the gastric wall. It is, however, a term often used loosely for clinical conditions associated with any upper gastrointestinal (GI) manifestations without specific clinical or radiological changes. In dogs, the sudden onset of vomiting suspected to be associated with gastric mucosal injury or inflammation is generally referred to as *acute gastritis.* The cause is commonly inferred from the clinical history, but the diagnosis is rarely confirmed by biopsy and the treatment is mainly symptomatic and supportive, rather than disease specific (Simpson, 2013).

Chronic gastritis may present clinically as a disease of acute onset, with progression to chronic gastritis because of persisting injury or sequela. This is the case in gastritis associated with long-term intake of some drugs (e.g. non-steroidal anti-inflammatory drugs; NSAIDs), severe stress (e.g. trauma, surgery), ischemia and shock, systemic infections and mechanical trauma (e.g. intubation-associated mucosal lesions). There is little evidence to support a role for any infection agent in acute gastritis (Brown *et al.*, 2007; Simpson, 2013).

Gastric foreign bodies are very common in dogs and tend to be seen in younger animals (Sullivan and Yool, 1998). If the foreign body becomes lodged in the pylorus, the animal will present with acute-onset clinical signs. However, in many cases, the object will

remain in the body of the stomach moving intermittently into the pylorus (Sullivan and Yool, 1998; Wilcock, 2013)

The International GI Standardization Group of the World Small Animal Veterinary Association (WSAVA) has provided guidelines for the normal histology of the stomach (Day *et al.*, 2008). The guidelines suggest that neutrophils should not be present in the normal canine gastric mucosa, so their presence generally suggests an acute inflammatory process which may or may not involve mucosal ulceration. However, there is one category of acute gastritis commonly found in the dog: acute non-erosive gastritis, in which diagnosis relies on the successful response to the symptomatic treatment.

2 – Gastric erosion or ulceration

Gastric ulcers are rarely primary. No breed, age or sex predilections are reported for canine gastric ulcer disease (Parrah *et al.*, 2013). If the ulcer is located in the stomach or in the proximal duodenum it is also called a “peptic ulcer” due to it being constantly bathed in pepsin. While erosions involve only the mucosa (Fig. 1A), ulcers can reach at least the level of the *muscularis mucosa* (Sullivan and Yool, 1998). However, for some pathologists this distinction is irrelevant, being merely indicative of the lesion depth. Therefore, an ulcer means “epithelial loss” and depending upon the depth, it can be superficial or deep and range from erosion to full thickness bleeding or perforating ulcer (Parrah *et al.*, 2013).

The classification of gastric ulcers as acute or chronic is more related to their clinical presentation than to their histological features. In animals, the aetiopathogenesis of gastric ulceration is often not identified. In many cases, gastric ulcers are associated with surprisingly little inflammation and most cases of gastritis do not have ulceration at all. Thus, the unequivocal classification of these lesions as acute or chronic is difficult from the histological perspective.

The pathophysiology of gastric ulceration is multifactorial and includes physical damage to the gastric mucosa, impairment of mucosal defence and chemical changes in the mucosa and in its repair process (Parrah *et al.*, 2013). The causes of canine gastric ulceration are reviewed in Table 1.

In dogs, mechanical abrasion is the most common cause of ulceration. Normally it is associated with the ingestion of a wide range of materials such as abrasive foods, household chemicals, common garden and woodland plants, items of clothing or household decorations. This type of ulceration is usually shallow and transient, completely healing

after a few hours (Wilcock, 2013). A few neutrophils may be found in the superficial *lamina propria* or intermingled with some fibrin and mucus covering the surface of the ulcer (Wilcock, 2013).

Some substances have been implicated in causing damage to the canine gastric mucosa including glucocorticoids such as dexamethasone (Rohrer *et al.*, 1999; Stanton and Bright, 1989; Valin and Allard, 2012) and a number of NSAIDs such as ketorolac (Mathews *et al.*, 1996). Cyclooxygenase (COX)-2 selective inhibitors (e.g., carprofen, etodolac, deracoxib, meloxicam) have been developed to minimize the ulcerogenic effect of NSAIDs in the GI tract. Although they minimize the risk, they do not completely eliminate it and many dogs have developed gastric ulceration following the administration of these putatively safer NSAIDs (Enberg *et al.*, 2006; Lascelles *et al.*, 2005). Some NSAIDs are especially well known for their ulcerogenic potential in dogs, including naproxen and flunixin meglumine (Dow *et al.*, 1990) or ibuprofen (Godshalk *et al.*, 1992; Smith and Taylor, 1993; Stanton and Bright, 1989). The ulcerogenicity of NSAIDs is related to their inhibition of the enzyme COX in the prostaglandin synthesis pathway, resulting in the loss of the gastric protective effects of prostacyclin and prostaglandin E (Enberg *et al.*, 2006).

The gross and microscopic appearance of ulcers varies with their severity and duration. Macroscopically, an ulcer is a round to oval mucosal defect, with a smooth base and perpendicular borders. Ulcers that develop acutely have regular and slightly raised borders (Fig. 1B). Progression to perforation is rare and usually requires persistence of the injurious stimulus (Fig. 1C) (Wilcock, 2013). In the chronic form, the borders are still regular, but become elevated and there are signs of inflammation in the surrounding mucosa (Fig. 1D). Ulcers that occur as a consequence of administration of NSAIDs are concentrated in the antropyloric area (Stanton and Bright, 1989). They are often small (<2cm in diameter) with a 'punched out' appearance and the walls are only slightly raised from the surrounding mucosa (Sullivan and Yool, 1998). Additionally, the adjacent mucosa may appear to have radial folds, as a result of parietal scarring. Distinctively, in the ulcerative cases of gastric cancer (GC) these lesions may have thickened and irregular mucosal margins, scirrhous change at the base and perhaps serosal thickening**.**

Microscopically, the base and margins of subacute to chronic ulcers are composed of granulation tissue of variable thickness and maturity, infiltrated by a mixed inflammatory cell population, and covered by a thin layer of necrotic debris. Chronic ulcers can fluctuate in size. Depending on the relative dominance of the reparative processes and the severity of ulceration, the layer of granulation tissue can be thick and mature or thinner and less mature, with superficial evidence of recent necrosis (Brown *et al.*, 2007).

Dogs subjected to a regular and intense exercise have a higher incidence of gastric ulcers (Chatelain *et al.*, 2014; Davis *et al.*, 2003b; Dennis *et al.*, 2008; Ritchey *et al.*, 2011) and gastric disease has been identified as a causative factor in cases of sudden death during dog sled races (Chatelain *et al.*, 2014; Davis *et al.*, 2003a; Dennis *et al.*, 2008). Possible explanations for this occurrence relate to the diet commonly used to support the extraordinary calorific demands of these athletes and in the stress of strenuous competition. A high-fat diet can lead to delayed gastric emptying and gastric hyperacidity, which, in isolation or together with the physiological response to stress (reflected in high levels of circulating cortisol) may predispose these animals to GI ulceration (Davis *et al.*, 2003a).

Gastric stress ulcers are poorly defined in the dog. Gastroduodenal ulceration has been reported in dogs in conjunction with severe burns, heat stroke, multiple trauma, head injuries and spinal cord disease (Dowdle *et al.*, 2003; Neiger and Simpson, 2000). However, these changes could be more related to the drugs used for the pain management and treatment of the inflammatory conditions than to the physiological response to stress under hospitalization.

Gastric ulceration is a frequent complication in dogs with hypoadrenocorticism. Systemic hypovolemia with an attendant decrease in gastric mucosal blood flow, loss of the permissive effect of glucocorticoids in mucosal defence, and significant electrolyte abnormalities are all likely contributors to the gastritis seen with this endocrinopathy (Henderson and Webster, 2006). It should be noted that, unlike people, dogs with renal failure rarely show gastric necrosis and ulceration (Peters *et al.*, 2005). In contrast, liver disease is considered one of the two most common risk factors for gastroduodenal ulceration in dogs and its pathogenesis is probably related to alter gastric blood flow due to portal hypertension, delayed epithelial turnover, gastric hyperacidity and hypergastrinemia (Henderson and Webster, 2006).

Animals with mastocytosis or mast cell tumours may have gastric ulcers (Howard *et al.*, 1969; Ozaki *et al.*, 2002) that can be multiple (Stanton and Bright, 1989). These tumours produce and release histamine, which binds to H2 receptors on gastric parietal cells constituting a powerful stimulant to acid secretion. Forty years ago, gastric ulcers were reported to occur in up to 80% of dogs with mast cell tumours (Howard *et al.*, 1969). However, in the authors opinion this value is no longer accurate and the actual incidence is much lower.

Infestation with *Physaloptera* spp. nematodes, including *P. rara* and *P. canis*, may be an infrequent cause of gastric ulceration associated with chronic vomiting in dogs (Burrows, 1983). The worms may be free in the lumen causing minimal inflammatory reaction (Pohlit, 2014), but more commonly attach to gastric mucosa where they feed on blood. They

occasionally change their attachment site resulting in multiple small bleeding wounds that become inflamed (Clark, 1990).

Despite the importance of *Helicobacter pylori (H. pylori)* as a cause of gastric ulcers in man (Marshall and Warren, 1984), no significant relationship has been demonstrated between *Helicobacter* spp. infection and gastric ulceration in dogs.

In man, peptic ulcer formation as a result of excessive gastrin production and hyperacidity caused by a gastrinoma is known as the Zollinger-Ellison syndrome. This is a condition often characterized by the triad of hypergastrinaemia, hypertrophic or atrophic gastritis and GI ulceration (Hayden and Henson, 1997; Simpson and Dykes, 1997). Few cases of a canine homologue of Zollinger-Ellison syndrome associated with gastric ulcer formation are reported (Fukushima *et al.*, 2004; Gal *et al.*, 2011). The gastrin production by these tumours directly stimulates secretion of hydrochloric acid from the parietal cells, as well as indirectly stimulating acid production by releasing histamine from fundic enterochromaffin-like cells, thereby compromising the protective mechanisms and reducing the epithelial cell turnover of the gastric mucosa (Hughes, 2006).

3 - Chronic Gastritis

Chronic gastritis is defined clinically as intermittent vomiting with duration of more than 1 – 2 weeks (Dowdle *et al.*, 2003). The diagnosis is based on the microscopic examination of gastric biopsy samples and is subclassified according to the histopathological changes and aetiology. For the histologic diagnosis of gastritis, an increase in mucosal leukocytes accompanied by other evidence of inflammation such as hyperaemia, oedema and lesions in the adjacent structural components of the mucosa (e.g., epithelial injury or reparative fibrosis) must be identified. Canine gastritis is commonly categorized according to the nature of the predominant cellular infiltrate (e.g., eosinophilic, lymphocytic, plasmacytic, granulomatous, lymphoid follicular), the presence of architectural abnormalities (e.g., atrophy, hypertrophy, fibrosis, oedema, ulceration, metaplasia), and their subjective severity (e.g., mild, moderate, severe) (Day *et al.*, 2008; Wilcock, 2013).

3.1 - Lymphoplasmacytic gastritis

Lymphoplasmacytic gastritis, with or without concomitant lymphoid follicular hyperplasia, remains the most common form of canine gastritis (CC Brown, 2007). It

may occur in isolation or together with enteritis and colitis, as part of the inflammatory bowel disease (IBD) syndrome. Chronic gastritis is seen predominantly in middle aged to older animals, although young animals may also be affected. There is no sex nor breed predisposition (Sullivan and Yool, 1998). Histologically, lymphoplasmacytic inflammation is characterized by infiltration of these cells into the *lamina propria* (Fig. 2A) and is often accompanied by distinct increases in the number of intraepithelial lymphocytes within both surface and deep epithelial structures (Day *et al.*, 2008). The cellular infiltrate varies widely in severity and it may be accompanied by mucosal atrophy or fibrosis, and less commonly hyperplasia (Simpson, 2013).

In accordance with WSAVA guidelines (Day *et al.*, 2008), the mucosa of the normal gastric body may have 0.5-13 (mean: 4.2) *lamina propria* lymphocytes and 0-5.83 (mean: 1.59) *lamina propria* plasma cells, while in the antrum, 0.5-13 (mean: 4.2) *lamina propria* lymphocytes and 0.5-15.5 (mean: 6.8) *lamina propria* plasma cells are considered normal. Another distinctive feature of this particular gastric inflammation is hyperplasia of the gastric lymphoid aggregates (Fig. 2B), which may sometimes be severe (Day *et al.*, 2008).

This form of canine gastritis may sometimes be associated with the presence of *Helicobacter* spp. organisms but there is no evidence supporting a causative role between this infection and gastric pathology.

In dogs with idiopathic lymphoplasmacytic gastritis, gastric atrophy correlates with the expression of mRNA for interleukin (IL)-1β and IL-10 and the presence of neutrophils. Furthermore, the histological severity of lymphoplasmacytic gastritis is correlated with atrophy, infiltration with lymphocytes and macrophages, and expression of genes encoding IL-10 and interferon (IFN)-γ (Wiinberg *et al.*, 2005).

3.2 - Eosinophilic gastritis

Eosinophilic gastritis is a less frequent condition of the canine stomach (Lidbury *et al.*, 2009), often believed to be a manifestation of a generalized GI tract hypersensitivity reaction (eosinophilic gastroenteritis) (Neiger, 2008). Dogs of ≤ 5 years of age are most commonly affected; there is no sex predisposition, and German shepherd dogs and rottweilers are more predisposed (van der Gaag, 1988). However, eosinophilic gastritis has also been identified in dogs as an isolated condition of the stomach alone (Hayden and Fleischman, 1977).

Macroscopically, eosinophilic gastritis may present as hypertrophy of the rugal folds with mucosal ulceration (Neiger, 2008) as well as thickening of the gastric wall, mucosal necrosis and haemorrhagic foci.

The diagnosis of eosinophilic gastritis requires an increased number of mucosal eosinophils and that eosinophils are the dominant inflammatory cell type. Other causes of eosinophilic infiltration of the gastric mucosa (e.g. parasitic disease) must be excluded. According to the WSAVA guidelines (Day *et al.*, 2008) , the mucosa of the normal gastric body and antrum can have 0-2 (mean: 0.5) and 0-6 (mean: 2.7) eosinophils per 10,000 μm^2, respectively. Eosinophils counts above these levels would suggest eosinophilic gastritis (Figure 2C). The eosinophilic infiltration is mainly limited to the gastric mucosa and rarely extends into the *muscularis mucosa* or serosa (Sattasathuchana and Steiner, 2014).

The cause of canine eosinophilic gastritis remains unknown, but factors such as genetic predisposition and diet are likely to be involved in its pathogenesis (Sattasathuchana and Steiner, 2014). Important differentials include gastric parasitic infections by *Physaloptera* spp. (Neiger, 2008), *Gnathostoma spp.* (Maleewong *et al.*, 1992), *Cryptosporidium spp.* (Ellis *et al.*, 2010) and *Heterobilharzia americana* (Rodriguez *et al.*, 2014) and eosinophilic infiltration accompanying mast cell tumours (Ozaki *et al.*, 2002).

Scirrhous eosinophilic gastritis is a rare variant of eosinophilic gastritis (Hayden and Fleischman, 1977) in which the stomach is enlarged and has a greatly thickened wall. Microscopically, there is marked infiltration of eosinophils, dense bands of fibrous tissue (granulation tissue and mature collagen) replacing most of the muscular layers and thickening the submucosa and serosa. The eosinophils are distributed in a linear-like to diffuse fashion, often forming perivascular aggregates, primarily within the muscular layers and serosa. Associated degeneration of the gastric arteries is evident ranging from fibrinoid necrosis to panarteritis. Gastric inflammation does not extend into the intestinal tract. Although the cause of this condition was not determined, its basis was probably immunological.

3.3 - Granulomatous gastritis

Granulomas may be present in the canine gastric mucosa in a variety of infectious diseases and as a reaction to endogenous substances and foreign objects, such as sewing needles (Pratt *et al.*, 2014). Endoscopic appearance ranges from non-specific minor changes to thickened mucosal folds with outlet obstruction. Histologically, the granulomatous lesions consist of the typical nodular, circumscribed collections of macrophages admixed with variable numbers of lymphocytes, eosinophils, multinucleated giant cells and neutrophils, with or without central necrosis and a peripheral lymphoid cuff (Ectors *et al.*, 1993).

Parasites that target the canine stomach are uncommon, but spirocercosis caused by the nematode *S. lupi* is one example. After ingestion, the larvae of this organism penetrate the gastric mucosa and provoke a severe mixed inflammatory/neoplastic-like response. These nodular foci are usually referred to as granulomas; however, some authors have

suggested that this designation is inappropriate (van der Merwe *et al.*, 2008). Initially the larvae are surrounded by highly vascularized loose connective tissue, which contains fibrin-rich fluid, neutrophils and foci of necrosis. Later, this tissue is composed mostly of actively dividing fibroblasts with an embryonic appearance, sometimes resembling a sarcoma (Bailey, 1963).

Another nematode that may infest the stomach of dogs is *Gnathostoma spinigerum*. Groups of up to 10 adults can be lodged in nodules in the submucosa, which may protrude into the gastric lumen. The wall of these nodules is comprised of granulation tissue and reactive fibrous stroma and in the centre the parasites are embedded in a purulent and haemorrhagic exudate. Focal granulomas may centre on eggs trapped in the connective tissue (Maleewong *et al.*, 1992). Another trematode parasite, endemic to the Gulf coast and south Atlantic region of the USA, *Heterobilharzia americana* (*H. americana*) was recently associated with gastric granulomatous inflammation circumscribing trapped eggs in one of 32 infected dogs (Rodriguez *et al.*, 2014).

Histoplasmosis is a relatively common systemic fungal disease of the dog caused by *Histoplasma capsulatum*. The infection is probably acquired via inhalation or ingestion of infective conidia. These are phagocytosed by macrophages and can be disseminated via the bloodstream or lymphatics to any organ system. The disease can be subclinical or cause clinical granulomatous disease affecting, among other organs, the canine GI tract (Bromel and Sykes, 2005; Lin Blache *et al.*, 2011). Young, large-breed, male dogs in the US Gulf States may develop mucosal and submucosal suppurating granulomas in the stomach and elsewhere in the GI tract, caused by the fungus *Pythium insidiosum* (Grooters and Gee, 2002). Additionally, cryptococcosis resulting in a granulomatous gastritis mimicking carcinoma has been reported in a Doberman pinscher as a cause of gastric outlet obstruction (van der Gaag *et al.*, 1991).

4 – Gastric mucosal fibrosis and atrophy

Atrophy of the gastric mucosa has been traditionally defined as “the loss of appropriate glands” (Rugge *et al.*, 2002) presumably following a chronic inflammatory process, since the mucosa becomes inactive and fibrosis replaces the normal glandular structures.

Endoscopically, the gastric mucosa appears discoloured and thin, with prominent blood vessels (Sullivan and Yool, 1998). In human mucosal atrophy, especially in severe cases, the gastric rugae can disappear from the fundus or body, while in the antrum, mucosal thinning can be grossly unapparent (Dixon *et al.*, 1996). In man, an inherited form

of atrophic gastritis typically confined to the gastric body and fundus is termed autoimmune chronic atrophic gastritis and is characterized by an immune response directed toward parietal cells and intrinsic factor (Neumann *et al.*, 2013) .This will result in the destruction of parietal cells, which leads to profound hypochlorhydria (and elevated gastrin levels), and in inadequate production of intrinsic factor that may leads to vitamin B12 malabsorption and pernicious anemia. In turn, the gastrin trophic effect exert on enterochromaffin-like cells is hypothesized to be one mechanism counteracting in the malignant transformation of these cells into carcinoid tumours (Neumann *et al.*, 2013).

Microscopically, chronic atrophic gastritis is the condition that combines mononuclear infiltration, glandular atrophy (Fig. 2D), regenerative glandular nesting and mucosal fibrosis. Lymphoid follicle hyperplasia may be present. There is a reduction in overall mucosal thickness and in most cases, an obvious reduction in the number of parietal cells with a corresponding increase in the proportion of mucus-producing epithelial cells within the gland (Wilcock, 2013). In dogs, atrophic gastritis is often associated with a marked cellular infiltrate (Rousseau, 2005). However, evidence that the pathogenesis of fibrosis is associated with previous inflammatory conditions is still lacking.

Atrophic gastritis has been infrequently found in dogs and there is no proof of its clinical significance (Simpson, 2013).

In one study of experimentally-induced intestinal metaplasia (IM) in the stomach of dogs, Wang and colleagues described expression of Bcl2 in precedent lesions of chronic atrophic gastritis, suggesting that this protein may be involved in the initial process of genetic instability and that disturbances in the regulation of apoptosis may constitute an initial step in the malignant transformation of the normal gastric mucosa (Wang, 2000). Atrophy of the gastric mucosa that may progress to adenocarcinoma has been reported in Norwegian Lundehund dogs (Berghoff *et al.*, 2007; Kolbjørnsen *et al.*, 1994) and in this breed a reduction in parietal cells and hyperplasia of neuroendocrine cells has been associated with gastric adenocarcinoma (Qvigstad *et al.*, 2008).

5 – *Helicobacter spp.* infection

The gastric mucosa of dogs is often colonized by *Helicobacter* spp. other than *H. pylori*. Non-*H. pylori* Helicobacters (NHPH) are present in 67-86% of clinically healthy dogs, in 61-100% of animals presenting with chronic vomiting (Geyer *et al.*, 1993; Hermanns *et al.*, 1995; Hwang *et al.*, 2002; Lecoindre P., 1995; Polanco *et al.*, 2011) and in 100% of laboratory dogs and dogs from local shelters (Eaton *et al.*, 1996; Henry *et al.*, 1987;

Simpson *et al.*, 1999). The predominant gastric *Helicobacter* spp. in dogs are *H. felis*, *H. bizzozeronii* and *H. heilmannii sensu stricto* (*s.s.*), while *H. salomonis* is less often detected and the prevalence of *H. cynogastricus* and *H. baculiformis* has not yet been studied (Baele *et al.*, 2009; Haesebrouck *et al.*, 2009; Haesebrouck *et al.*, 2011; Van den Bulck *et al.*, 2006). However, *H. pylori* has occasionally been recognized in the canine stomach (Buczolits *et al.*, 2003; Chung *et al.*, 2014; Ekman *et al.*, 2013; Taulescu *et al.*, 2009). Mixed infections with different species can also occur (Ekman *et al.*, 2013; Van den Bulck *et al.*, 2005) and the number of organisms present can be variable.

While many studies have reported that the fundus and body have higher bacterial density and a higher probability of finding *Helicobacter* spp. (Anacleto *et al.*, 2011; Simpson *et al.*, 1999; Yamasaki *et al.*, 1998) others have found no significant differences between the density of NHPH in the fundus, body and antrum (Buczolits *et al.*, 2003; Cattoli *et al.*, 1999; Gombač *et al.*, 2010; Prachasilpchai *et al.*, 2007; Recordati *et al.*, 2009) of the canine stomach. The discrepancies in these results can be attributed to the different laboratory diagnostic methodologies used by the various research groups. Canine NHPH colonize the superficial mucus layer and gastric glands and may also be observed intracellularly (Fig. 3). Vacuolation and necrosis of parietal cells has also been observed in the gastric mucosa of dogs naturally infected by *Helicobacter* spp. (Fig. 3B) (Happonen *et al.*, 1996; Hermanns *et al.*, 1995; Takemura *et al.*, 2013; Yamasaki *et al.*, 1998).

In contrast, in man, *H. pylori* does not affect the parietal cells or the deep gastric glands, being located more superficially and primarily affecting the antrum. Furthermore, the characteristic human *H. pylori* surface epithelium and foveolar neutrophil infiltration and “pit abscess” formation (Fig. 3D) (Dixon *et al.*, 1996) is not usually seen with NHPH infection in the canine stomach. Presumably, in the acute phase, the infection briefly triggers trivial initial signs and goes unnoticed, only being detected later as an occasional finding or due to the persistence or deterioration of the animal’s clinical signs. In dogs, the inflammation is generally mononuclear in nature and ranges from mild to moderate in severity. Thus, gastritis associated with NHPH is mostly less active, compared with *H. pylori*-associated gastritis.

Although *H. pylori* is well accepted as an important human pathogen and is believed to be the primary cause of chronic gastritis, gastric and duodenal ulceration, and even gastric carcinoma and mucosal-associated lymphoid tissue (MALT) lymphoma, the pathogenic significance of gastric *Helicobacter* species in dogs is debatable. Histological findings, such as mild to moderate gastritis, reduction in mucus production, glandular degeneration, interstitial oedema, fibrosis of *lamina propria,* hyperplasia, vacuolation, and necrosis of parietal cells were described in dogs naturally infected by *Helicobacter* species (Happonen *et al.*, 1996; Hermanns *et al.*, 1995; Takemura *et al.*, 2013; Yamasaki *et al.*, 1998).

Peyrol *et al.* (1998) found no pathogenic significance for *H. bizzozeronii* in the canine stomach (Peyrol *et al.*, 1998). Simpson *et al.* (1999) found no significant difference in clinical symptoms, inflammation and gastric function between control and experimentally *H. felis* infected dogs. (Simpson *et al.*, 1999). Wiinberg *et al*. (2005) have quantitatively examined mucosal inflammatory and immune responses in dogs with gastritis and assessed the relationship of these responses to infection with *Helicobacter* spp. (Wiinberg et al., 2005). They concluded that gastric infection by NHPH in dogs was associated with increased expression of transforming growth factor (TGF)-β and fibrosis. However, no significant associations between *Helicobacter* spp. infection and proinflammatory cytokine expression, severity of gastritis or differences in the pathogenicity of the different *Helicobacter spp.* were found. Another investigation documented no significant change in epithelial proliferation or E-cadherin expression in the gastric mucosa of dogs infected with *Helicobacter spp.* (Takemura *et al.*, 2013). Additionally, canine *H. heilmannii* infection was found to be associated with increased numbers of lymphoid follicles (Takemura *et al.*, 2013). In the majority of dogs infected with NHPH, the limited inflammatory response and the absence of clinical signs suggests that these are commensal rather than pathogenic organisms, although seroconversion and hyperplasia of the lymphoid follicles indicates that significant antigenic stimulation occurs (Simpson, 2013). Nevertheless, the presence of NHPH in the canine stomach in conjunction with more severe pathologies has been documented. For instance, *Helicobacter spp.* organisms were found in all cases of canine gastric polyps (Taulescu *et al.*, 2014) and Poutahidis and colleagues detected moderate numbers of large *Helicobacter*-like organisms in all parts of the glandular stomach of a Poodle affected by a choriocarcinoma (Poutahidis *et al.*, 2008). To the best of our knowledge, so far there are no evidences reporting the occurrence of gastric lymphoma and concomitant presence of *Helicobacter spp.* in the dog.

Comparative analyses between the available genome sequences revealed that canine NHPH lack all known *H. pylori* adhesins (Haesebrouck *et al.*, 2009). Although both *H. pylori* and NHPH share virulence factors involved in epithelial cell death such as the ɣ-glutamyl transpeptidase, all NHPH lack cytotoxin-associated genes, pathogenicity islands and a functional vacuolating cytotoxin A.

A recent study has characterized the glycosylation profile of the normal canine gastric mucosa, with focus on the expression of histo-blood group antigens (Amorim *et al.*, 2014b). The canine gastric mucosa lacks type 1 Lewis antigens, but has extensive expression of type 2 Lewis structures and A antigen, both in the surface and glandular epithelium. Furthermore, to determine if this glycosylation profile was somehow related to the host-adaptation of the different *Helicobacter* spp., their binding capacity to canine gastric mucosa was evaluated: *H. heilmannii s.s.* had the highest adhesion scores in the canine antral mucosa, while the SabA-positive *H. pylori* strain showed the highest adhesion

in the body region. It was concluded that the differences in the glycosylation profile between dogs and man, together with evidence that *H. pylori* and NHPH strains display different abilities to adhere to canine gastric mucosa, indicate that alternative glycan receptors may be involved in *Helicobacter* spp. adhesion and may explain the differences between the prevalence of gastric helicobacters in both mammalian species.

6 – Gastric proliferative disease

6.1 - Hypertrophic gastritis

Giant hypertrophic gastritis is a rare condition in the dog and, as the denomination suggests, is characterised by a marked thickening of the gastric wall and hypertrophy of the gastric mucosa, causing a cerebriform appearance of the rugal folds protruding into the gastric lumen (Figure 4B) (Kipnis, 1978; Krunngen, 1977; Rallis *et al.*, 2007; Slappendel *et al.*, 1991; van der Gaag *et al.*, 1976; Vaughn *et al.*, 2014). The fundus and body of the stomach are affected and the pyloric antrum is often spared (Guilford, 1996; Vaughn *et al.*, 2014). The majority of dogs reported with giant hypertrophic gastritis are of the Drentse Patrijshond and Bansenji breeds; however, the disease is also reported in a 7-year-old male Boxer (van der Gaag *et al.*, 1976), a 6-year-old female of undetermined breed (Kipnis, 1978), an 11-year-male Old English Sheepdog (Rallis *et al.*, 2007); three 10-11-year-old Cairn terriers (Munday *et al.*, 2012) and a 4-year-old neutered male Jack Russell terrier (Vaughn *et al.*, 2014). A polypoid variant was documented in a 7-year-old male West Highland white terrier (Lecoindre *et al.*, 2012).

Microscopically, tissues from the fundus and body stomach have similar features. The mucosa displays marked foveolar and glandular hyperplasia and dilation. Mild mononuclear inflammatory infiltration (i.e. plasma cells, lymphocytes, and macrophages) is present in the *lamina propria* (Fig. 4C). Cuboidal or flattened epithelial cells often cover the dilated glands. Another characteristic histopathological finding is hyperplasia of the mucous cells with concurrent loss of chief and parietal cells (Lecoindre *et al.*, 2012).

In Basenji dogs, a variant of this disease was described and includes the above findings together with small intestinal lymphoplasmacytic enteritis (Krunngen, 1977) and consistent hypoalbuminemia and hyperglobulinemia (Breitschwerdt *et al.*, 1983). Additionally, hypertrophic gastritis has also been described in the Drentse Patrijshond breed as part of a condition termed familial stomatocytosis-hypertrophic gastritis (Slappendel *et al.*, 1991). In this case, the gastric disease is accompanied by other signs including stomatocytosis, progressive liver disease, cystic kidney disease, and polyneuropathy (Slappendel *et al.*,

1991). Since in this later breed only the pylorus was affected, some authors have suggested that these animals were probably suffering from a distinct disease process (Vaughn *et al.*, 2014), also described elsewhere in this manuscript.

Human Ménétrier's disease closely resembles canine giant hypertrophic gastritis. It is characterized by marked rugal hypertrophy in the proximal portion of the stomach (body and fundus), hypoalbuminemia and unique histological findings: often marked foveolar hyperplasia of the gastric mucosa with glandular or oxyntic atrophy and cystic dilatation; inflammatory infiltration and accumulation of eosinophils can be present in the *lamina propria*; and thickening and disorganization of the *muscularis mucosae*, often appearing as strands of smooth muscle extending into the *lamina propria* (Coffey and Tanksley, 2012). Some investigators have questioned however the importance of the inflammatory infiltrate, suggesting that Ménétrier's disease simply represents a non-inflammatory gastropathy (Wolfsen *et al.*, 1993). The same question was raised by both the group of Rallis (2007) and Vaughn (2014) when faced with the lack of inflammatory infiltration in the canine disease (Rallis *et al.*, 2007; Vaughn *et al.*, 2014).

Although the pathogenesis of this condition in the dog remains poorly elucidated, the development of this disease in three siblings suggests an inherited predisposition (Munday *et al.*, 2012). These three dogs also developed GC as has been documented in human Ménétrier's disease (Sundt *et al.*, 1988). The coexistence of these two types of lesions in dogs was reported twice and a possible link between recurrent gastric inflammation and neoplastic transformation was proposed (Lecoindre *et al.*, 2012).

An association between human Ménétrier's disease and overexpression of epidermal growth factor receptor (EGFR) in the gastric mucosa due to local overproduction of TGF-α has been reported (Coffey and Tanksley, 2012). In initial clinical trials, human patients were treated with a monoclonal antibody that inhibits vascular endothelial growth factor receptor signalling (Cetuximab®) and showed some clinical improvement (Coffey and Tanksley, 2012).

7 – Gastric polyps

Gastric polyps are considered to be a heterogeneous entity broadly defined as luminal lesions projecting above the plane of the mucosal surface that can be biologically classified as non-neoplastic and neoplastic lesions.

In dogs, non-neoplastic gastric polyps are rare and usually affect older animals. The gastric antrum appears to be a preferential location for polypoid growths (Figure 4D) and the hyperplastic histological-type (described below) is encountered most often (Taulescu *et al.*, 2014).

The World Health Organisation (WHO) classification of tumours in domestic animals presents a simple scheme where only two entities are recognized: hyperplastic (regenerative) and inflammatory (benign lymphoid) polyps (Head, 2003). The former are histologically identical to the human homonymous lesion and consist of hyperplastic, elongated and branching foveolae, set in an abundant oedematous and inflamed stroma. Cystic dilation of the pits is almost invariably present in the deeper portions and the surface is lined by a single-layer of foveolar-type epithelium. Inflammatory polyps are defined by the presence of normal epithelium covering granulation tissue that is infiltrated by a variety of inflammatory cells (Fig. 4E), sometimes accompanied by lymphoid aggregates with prominent germinal centers (Taulescu et al., 2014). In veterinary medicine, the lack of invasion across the basement membrane and into the *lamina propria* or submucosa is the only reliable criterion for distinguishing a gastric polyp from a well differentiated papillary adenocarcinoma (Head, 2003).

The pathogenesis of gastric polyps is still unknown, but studies suggested a possible hereditary predisposition in the French bulldog (Gualtieri *et al.*, 1999; Kuan *et al.*, 2009). Canine gastric polyps may occur against a background of chronic gastritis and it is considered that these lesions develop as the result of an exaggerated mucosal response to injury. An association with *Helicobacter spp.* infection has also been reported (Taulescu *et al.*, 2014). The presence of *Helicobacter spp.*, the cellular proliferative activity, potential phenotypic alterations and COX-2 and p53 expression in a series of canine spontaneous gastric polyps has been investigated. These studies showed normal expression of almost all of these factors and reduced proliferative activity, consistent with these lesions being benign in nature (Amorim *et al.*, 2014c).

Additionally, hamartomatous and heterotopic types are recognized entities in man and one case of canine hamartomatous polyp is described (Taulescu *et al.*, 2014) .

Neoplastic gastric polypoid lesions include adenomas, defined by the WHO as circumscribed polyps composed of tubular and/or villous structures lined by dysplastic epithelium (Fig. 4F). The main histological findings in gastric adenomas when compared with normal gastric mucosa are (1) progressive loss of differentiation in the upper portions of the gland with branching crypts and a patchy increase in cell layers, (2) progressive change in cell shape and size, and (3) the nuclei are larger round, central and hyperchromatic with larger nucleoli and more mitoses. These changes are present along the whole length of

the gland. Scanziani and colleagues reported the presence of argyrophil cells, identified by means of the Grimelius stain in all five cases tested and concluded that endocrine cells are present in a significant proportion in canine gastric adenomas (Scanziani *et al.*, 1993). Terragni *et al.* (2014) documented EGFR and Her-2 positive immunolabelling in three of five gastric adenomas and all of these lesions showed a wild type *KRAS* gene status.

8 – Special forms of gastropathy

8.1 - Hypertrophic pyloric gastropathy

Hypertrophic pyloric gastropathy is one of the terms coined for the syndrome of pyloric obstruction in dogs that can be caused by (1) hypertrophy of the circular muscle of the pylorus, (2) hyperplasia of the antropyloric mucosa, or (3) a combination of both changes.

The first form of the disease is the least common and is usually associated to congenital anomalies in Boxers, Bulldogs and Boston Terriers (Guilford, 1996; Peeters, 1991). In contrast, adult forms affect mainly male brachycephalic, middle-aged or older, small-breed dogs such as Shih Tzus, Lhasa Apso, Pekingese and Maltese, which can present with either the first or the third manifestation of the syndrome (Bellenger *et al.*, 1990; Guilford and Strombeck, 1996; Guilford, 1996; Peeters, 1991; Walter *et al.*, 1985). Mucosal hypertrophy alone is the most common lesion (Fig. 4A) and displays features in common with hypertrophic gastritis. If there is concurrent muscular hypertrophy, this is reflected on the cut surface of the pylorus by irregular firm thickening of the circular layer of the tunica muscularis. Microscopically, smooth-muscle fibres in affected fascicles are irregularly hypertrophic.

The pathogenesis of this condition is speculative and it is not clear whether muscular hypertrophy is primary or secondary. Neuroendocrine (hypergastrinemia), stress-related, (many of the cases are observed in highly excitable and nervous small breeds) or response to excessive mucosal growth are possible causes. However, since mucosal and muscular lesions can be present independently, they may have separate causes. Gastric antral hypertrophy could be another result of prolonged elevation of circulating gastrin, as a consequence of a canine gastrinoma (Gal *et al.*, 2011).

8.2 - Uremic gastropathy

Uremic gastritis was first described in 1934 as a result of 135 autopsies performed in uremic human patients (Jaffe and Laing, 1934). In dogs, gastric complications of uremia appear to be less common and less severe than in man (Peters *et al.*, 2005)

In 1979, Cheville provided a detailed description of the gastric pathological findings in a small group of uremic dogs with renal amyloidosis and pyelonephritis. At gross examination, changes were restricted to the fundic and body regions and included thickened rugae, oedema, necrosis, haemorrhage and ulceration (Cheville, 1979).

Canine uremic gastropathy is defined by oedema and mineralization of the *lamina propria*, atrophy of the gastric glands and vascular injury with thrombosis (Cheville, 1979; Peters *et al.*, 2005). Typically there is a horizontal band of acute epithelial necrosis affecting mostly the parietal cells in the middle third of the mucosa. In severe cases there is mineralization of basement membranes of the glands and of degenerate smooth muscle in the *muscularis mucosa* or tunica muscularis (metastatic calcification presumed). Mineralization of submucosal blood vessels may become extensive, involving the full thickness of the vessel wall and accompanied by medial necrosis and endothelial destruction (Peters *et al.*, 2005). According to (Peters *et al.*, 2005), mucosal necrosis and ulceration are uncommon findings in this condition so the designation of “ulcerative or erosive gastritis” on the basis of extrapolation from the human disease may not be appropriate. Presence of amyloid in the stomach of dogs with renal disease was also observed.

9 – Gastric metaplastic changes

Metaplasia is defined as a potentially reversible change from one fully differentiated cell type to another, implying certain adaptations to environmental stimuli and in which embryological commitments can be reversed under certain circumstances (Tosh and Slack, 2002).

9.1 - Osseous metaplasia/heterotopic bone formation

Osseous metaplasia or heterotopic bone formation is rare in the GI tract. In man, this change is usually associated with malignant lesions (Olinici *et al.*, 2002; Wilsher, 2011), Peutz–Jeghers syndrome (Narita *et al.*, 1995), inflammatory colonic polyps (Oono *et al.*, 2010), gastric adenomatous polyps of the stomach (Zapata *et al.*, 2012) and is found within

hyperplastic gastric polyps (Hayashi *et al.*, 1989; Ohtsuki *et al.*, 1987).

The pathogenesis of osseous metaplasia in the GI tract remains poorly understood. According to several investigations, osseous metaplasia has been associated with inflammatory changes and mucin production (Alper *et al.*, 2000; Haque *et al.*, 1996), local ischemia and/or necrosis (Ansari *et al.*, 1992). According to some theories, it may be either the inflammation that induces the osteogenic stimulation or perhaps local or unknown osteogenic factors secreted by the lesion that stimulate osteoblasts (Zapata *et al.*, 2012).

One case of gastric carcinoma with osseous metaplasia is described in a dog (Amorim, 2012). In this case, multiple foci of heterotopic ossification, consisting of osteoid and fully mature bone, surrounded by neoplastic epithelial cells were identified in the mucosa and muscular layers of the gastric body (Figure 5A). Immunoexpression of bone morphogenetic protein (BMP)-2/4, was observed both in the neoplastic cells and osteoblasts suggesting that, in this case, an osteogenic signal originated from the neoplastic epithelial cells and may have induced the differentiation of epithelial, mesenchymal or stem cells into osteoblasts. However, the reason why some tumours undergo osseous metaplasia while others do not is unknown, but is probably a result of a random activation with very little growth advantage for the neoplastic cells (Kypson *et al.*, 2003). Otherwise, osseous metaplasia would be more common.

9.2 - Glassy cell change

In 1991, Rubio *et al.* identified a new phenotype of glands in the human pyloric mucosa which were termed “glassy cells”. Glassy cells have a pale or eosinophilic cytoplasm with a ground-glass appearance, which pushes the nucleus towards the free border of the cell. These findings, which can affect one gland or a group of glands, can be misinterpreted as neoplastic ‘signet ring’ cells (Hughes *et al.*, 2011; Rubio *et al.*, 1991). Glassy cells were also detected in the gastric mucosa of baboons (Rubio *et al.*, 2008; Rubio and Hubbard, 1998). In a comparative study, these cells were found more frequently in patients residing in the Pacific basin than in those from Atlantic basin (Rubio *et al.*, 2005), so it was suggested that environment, rather than genetic factors, may influence the histological composition of the gastric mucosa (Rubio *et al.*, 2005). The same group reported glassy cells in the gastric mucosa of transgenic mice suggesting that this phenotypic alteration may also be induced by genetic manipulation (Judd *et al.*, 2005).

Previous studies showed that glassy cells were present in the mucosa of human cases of gastric carcinoma, peptic ulcer or other gastric diseases (Rubio *et al.*, 1991; Rubio *et al.*, 2005). These were also identified in lesions that often precede epithelial dysplasia

and carcinoma, such as Barrett's mucosa in oesophageal biopsies of baboons and people (Rubio, 2009; Rubio *et al.*, 2009).

Glassy cells have also been identified in the canine stomach (unpublished observations). Cells with the phenotype of human glassy cells were noted in the stomach of a 12-year-old male German shepherd dog, mainly in the transitional area between the oesophagus and the cardia. This is consistent with previous primates studies in which GCs were identified in different areas of the stomach (Rubio *et al.*, 1991; Rubio *et al.*, 2008). The canine glassy cells had large and glassy cytoplasm with nuclei displaced towards the luminal aspect of the gland. They were organized in clusters that mainly retained glandular architecture (Fig. 5B). This dog had a poorly cohesive carcinoma apparently originating in the gastric fundus and progressing towards the oesophagus. The significance of these cells in the canine gastric mucosa remains to be further elucidated.

9.3 - Mucous (pseudopyloric) metaplasia

Mucous metaplasia involves metaplastic transformation of the fundic-oxyntic mucosa to a mucus cell form. Normal oxyntic mucosa has straight glands composed of tightly packed chief cells, parietal cells, endocrine cells, and mucus cells with a higher ratio of glands to foveola compared with the antrum. It is postulated that continuous inflammation and atrophy leads to progressive loss of parietal cells and eventually the oxyntic mucosal glands come to resemble the antral/pyloric glands (pseudopyloric metaplasia) (Fig. 5C).

Pseudopyloric metaplasia was reported in 17 dogs that mostly had simple diffuse or atrophic forms of gastritis (van der Gaag, 1988).

9.4 - Intestinal metaplasia (IM)

Originally described in the 19th century, gastric IM is defined as the transformation of the foveolar and/or the glandular epithelium of the gastric mucosa by intestinal-like epithelium with goblet cells, enterocyte-like cells and Paneth cells. The last two cell phenotypes are absent in the so-called incomplete IM (Rubio, 2007).

Human IM is often encountered in chronic gastritis following *H. pylori* infection, which leads to gastric atrophy, progression to IM and, ultimately, GC (Correa, 1992). The variable outcome of *H. pylori* infection suggests that IM results from distinct adaptive responses in adverse circumstances, with the interplay between individual genetic variation and environmental exposures determining individual susceptibility (Barros *et al.*, 2010). In

man, about 80% of gastric carcinomas appear in the context of IM thus, IM is considered to be a preneoplastic condition, resulting in an increased risk for subsequent development of cancer (Uemura *et al.*, 2001).

Advances in molecular biology revealed that intestinal transcriptional factors regulate the proliferation and differentiation of intestinal epithelial cells by the expression of the main homeobox transcription factor CDX2 (Almeida *et al.*, 2003; Eda *et al.*, 2002; Silberg *et al.*, 2002). Under normal conditions, human CDX2 expression is restricted to the intestine, but it becomes expressed ectopically in human gastric IM lesions (Almeida *et al.*, 2003; Eda *et al.*, 2002). Two major pathways for the development of IM are proposed: (1) activation of signalling pathways normally absent from the stomach, but involved in intestinal development and differentiation, or (2) inactivation of pathways involved in establishing and maintaining the gastric phenotype. Moreover, it is hypothesized that *H. pylori* infection and consequent gastric inflammation provide the initial trigger for CDX2 expression in human gastric cells by inducing an altered signalling network. This will result in a certain level of CDX2 overexpression, which will persist via an autoregulatory mechanism that precipitates the subsequent cellular events (Barros *et al.*, 2010). Although these CDX2 regulatory mechanisms are insufficient to explain the maintenance of CDX2 expression, especially after eradication of infection and resolution of inflammation, the low reversibility of IM observed in human lesions suggests that another level of complexity is present in CDX2 regulation *in vivo* (Barros *et al.*, 2010).

IM is well-known and extensively studied in man and has been induced experimentally in some animal models including in gerbils, rats and dogs (Hashimoto *et al.*, 1980; Li *et al.*, 1994; Wang, 2000; Watanabe *et al.*, 1992). The Hashimoto group (1980) successfully induced IM in the stomach of Beagle dogs treated with N-methyl-N-nito-N-nitrosoguanidine (MNNG); however, this observation occurred after a long-term treatment, since IM was only achieved at the 128th experimental week.

Wang (2000) also induced IM in Beagle dogs, but in a shorter period of time by combining long-term oral administration of a small dose of MNNG and ranitidine plus intermittent low-dose localized X-irradiation of the stomach. Similar to the changes in man, the transition from canine NGM to IM involved several progressive steps including superficial gastritis, chronic atrophic gastritis, slight focal IM and moderate or severe IM (Wang, 2000). Furthermore, there was expression of APC, Bcl2 and Kras in the lesions of canine IM, suggesting that these tumour-related proteins may play a role in the malignant transformation of IM. Additionally, no p53 expression was detected in these induced canine IM lesions.

To the best of our knowledge, spontaneous development of gastric IM has never

been documented in normal or inflamed canine stomach but has been described in association with canine hyperplastic gastric polyps (Taulescu *et al.*, 2014). Additionally, in all cases of canine gastric adenocarcinoma, CDX2 expression has been recorded in foci of metaplastic change which included numerous goblet cells (Fig. 5D) (Doster *et al.*, 2011). Eventually, under certain pathological circumstances some changes may occur in the canine GI microenvironment that probably lead to IM and similarly to that seen in man, the upregulation of CDX2 may also be involved in this pathway (unpublished observations).

10 – Gastric dysplasia

10.1 - Epithelial dysplasia

The diagnosis of gastric dysplasia in animals is based on concepts described in human medicine, but is hampered by factors including (1) lack of a uniformly recognized classification, (2) significant interobserver variability in microscopic diagnosis, (3) absence of well-defined dysplastic features associated with particular entities of gastric carcinoma, and (4) a limited understanding of the clinical significance of a diagnosis of dysplasia (Lauwers and Riddell, 1999).

Microscopically, gastric epithelial dysplasia is characterized by a set of histological alterations based on cytological and architectural abnormalities. The cytological changes include cytoplasmic mucin depletion; cellular crowding and pleomorphism; nuclear hyperchromatism, pleomorphism and stratification; increased nuclear:cytoplasmic ratio, and increased mitotic activity. The architectural changes include glandular disorganization or budding, with irregular branching and dilatation and intraluminal folding. However, the unequivocal distinction between regenerative changes and dysplasia in gastric tissues remains difficult for veterinary pathologists.

In accordance with the Vienna classification, human dysplastic changes may be low-grade or high-grade (Schlemper *et al.*, 2000). Low-grade dysplasia is characterized by preservation of the glandular architecture, sometimes with the presence of pseudovilli, cystically dilated or slightly irregular glands with discrete intraluminal papillary projections or a serrated appearance. Glands are composed of tall and crowded cells, with or without mucus vacuoles. The nuclei are discretely pleomorphic, elongated, stratified and are located in the lower half of the cytoplasm. Mitotic index is low. High-grade epithelial dysplasia is characterized by marked distortion of the glandular architecture with crowded, irregular and ramified glands and frequent papillary intraluminal projections lined by stratified epithelium,

exhibiting pleomorphic nuclei that may overlap. There is marked mitotic activity and loss of normal cell polarity (Fig. 5E).

10.2 - Globoid Dysplasia

"Tubule neck dysplasia", "non-metaplastic dysplasia" or "globoid dysplasia" (GD) is a subtle alteration occasionally found in human gastric non-metaplastic mucosa (Ghandur-Mnaymneh *et al.*, 1988) and which is believed to be a precursor lesion of signet ring carcinoma (Ghandur-Mnaymneh *et al.*, 1988; Misdraji and Lauwers, 2002). In GD, the tortuous tubules have crowding of the neck region by enlarged clear cells lining the foveolae. The most distinctive morphological characteristic of GD is the presence of abundant vacuolated cytoplasm with excessive amounts of mucin, which compress the eccentric and hyperchromatic nuclei, giving an appearance similar to signet ring cells (Ghandur-Mnaymneh *et al.*, 1988; Misdraji and Lauwers, 2002). An immunohistochemical analysis of six cases of human gastric GD showed that the expression profile was MUC5AC (+), Ki-67 (+), p53 (+), p27 (+), p16 (+), MUC6 (-), Na^+K^+ ATPase (-) and E-cadherin expression with reduced intensity (Lino-Silva *et al.*, 2013).

GD has not been documented in the dog, but similar changes were noted in the antrum of a 12-year-old male crossbred Poodle (unpublished observations). This dog had generalized and diffuse foveolar hyperplasia with tufting of the superficial epithelium consistent with globoid change. The alteration was characterized by distinctive and increased retention of mucin in the supranuclear cytoplasm without nuclear atypia or disruption of the epithelial structures (Figure5F). High numbers of NHPH were also present in the stomach of this dog. No signs of neoplastic transformation were identified. However, similar lesions were reported in the adjacent non-neoplastic mucosa of patients diagnosed with hereditary diffuse gastric cancer (Carneiro *et al.*, 2004). Further studies are warranted in order to understand the implications of this histological alteration in the canine gastric mucosa.

11 – Gastric epithelial neoplasia

11.1 - Epidemiological features of canine gastric carcinoma (GC)

Gastric cancer accounts for < 1% of all reported neoplasms in dogs (Arnesen K, 2001; Crow, 1985; Patnaik *et al.*, 1977; Sullivan *et al.*, 1987). Carcinoma is the most frequent gastric tumour, comprising 50 –90% of all canine gastric malignancies and usually results in death (Patnaik *et al.*, 1977; Sautter and Hanlon, 1975; Swann and Holt, 2002).

Most canine GCs are located in the lesser curvature and pylorus, often progressing to involve most of the stomach body (Fonda *et al.*, 1989; Patnaik *et al.*, 1977; Sautter and Hanlon, 1975; Scanziani *et al.*, 1991; Swann and Holt, 2002). The reported age range of dogs with GC is 3 to 16 years (mean: 7.5 years) (Fonda *et al.*, 1989; Patnaik *et al.*, 1977; Scanziani *et al.*, 1991; Sullivan *et al.*, 1987; Swann and Holt, 2002) and, as in man, a higher incidence in males is reported (Carrasco *et al.*, 2011; Fonda *et al.*, 1989; Patnaik *et al.*, 1977; Scanziani *et al.*, 1991; Swann and Holt, 2002).

Breed predisposition to GC in Belgian Shepherd dogs (Tervuren and Groenendael) (Fonda *et al.*, 1989; Lubbes *et al.*, 2009; Sautter and Hanlon, 1975; Scanziani *et al.*, 1991; Seim-Wikse *et al.*, 2013), Rough Collies (Seim-Wikse *et al.*, 2013; Sullivan *et al.*, 1987), Staffordshire Terrier (Sullivan *et al.*, 1987), Chow Chows (Bilek and Hirt, 2007; Penninck *et al.*, 1998) and standard Poodles (Seim-Wikse *et al.*, 2013) are reported. In the author's institutions, sled dogs (e.g. Alaskan malamute, Siberian husky, Pyrenean Mountain dog) and Japanese Akitas are often affected (unpublished data); however, in determining any canine breed predisposition, the popularity of the breeds in the geographical location must be considered.

The incidence of GC is lower in dogs than in man (Crow, 1985; Fonda *et al.*, 1989), but in recent years this disease appears to be diagnosed more frequently. This likely reflects the use of more accurate diagnostic techniques such as endoscopy. Despite this, canine GCs are often diagnosed at an advanced stage, resulting in a poor prognosis and limited treatment options. A median survival time of 35 days has been described (Swann and Holt, 2002) and in about 70-90% of cases, the tumours have already metastasized at the time of diagnosis or death (Scanziani *et al.*, 1991; Sullivan *et al.*, 1987; Swann and Holt, 2002). Metastatic sites include the gastric lymph nodes, omentum, liver, duodenum, pancreas, spleen, oesophagus, adrenal glands, and lungs (Head *et al.*, 2008).

Most canine GCs 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 (Fig. 6A) (Guilford, 1996).

11.2 - Classification of gastric cancer

A. Early and advanced gastric carcinoma

Early gastric carcinoma (EGC) is defined as an invasive carcinoma confined to the mucosa and/or submucosa, with or without lymph node metastases, irrespective of the tumour size. In man, most EGCs are small (2-5 cm in size), located in the lesser curvature and seen endoscopically as polypoid (pedunculated or sessile), slightly elevated, flat,

slightly depressed or excavated lesions. Histologically, the most common forms of EGC are well differentiated, mostly with tubular and papillary architecture.

Despite advances in veterinary diagnosis and treatment, the early stages of GC are often asymptomatic and the late onset of clinical signs means that canine cases carry a poor prognosis in comparison with human patients, in which the prognosis of this condition is often excellent (Uedo *et al.*, 2006). In contrast, a recent publication described the successful treatment of EGC in a Shih-Tzu dog (Lee *et al.*, 2014). The rarity of this lesion in veterinary medicine inhibits more detailed investigation and classification. In human medicine, the Paris classification (2005), which focuses on the endoscopical categorization of superficial lesions in GI tract, remains a clinically important tool that helps to decide if neoadjuvant (pre-operative) therapy is necessary.

Advanced gastric carcinoma (AGC), which invades into the tunica muscularis or beyond, carries a worse prognosis. These lesions can be grossly exophytic, ulcerated, infiltrative or combined. The Borrmann's classification is usually employed for the macroscopic evaluation of human AGC. Histologically, these tumours often exhibit marked architectural and cytological heterogeneity. Due to the advanced stage of disease at the time of diagnosis, the great majority of canine GCs would be included in this stage.

B. Histological classification of gastric carcinoma

According to the WHO classification for domestic animals (Head, 2003), gastric carcinomas are divided into three categories based on the histopathological features including adenocarcinoma, squamous cell carcinoma and undifferentiated carcinoma. Adenocarcinomas are subdivided into papillary, tubular, mucinous and signet ring cell types, depending on the predominant histological picture and the principal cell type of the tumour (Head and Ring, 2003).

The human Lauren classification for GC is based on the architectural and cytological features of the tumour and has been applied successfully in previous investigations in the dog (Fonda *et al.*, 1989; Janke *et al.*, 2010; Patnaik *et al.*, 1978). Generally, papillary and tubular variants are classified into intestinal, expanding, or differentiated type, while mucinous and signet ring cell variants are categorized into diffuse, infiltrative, or undifferentiated type (Lauren, 1965).

In dogs, tubular adenocarcinoma is considered the most common histological subtype (Wilcock, 2013) (Figure 6B). Macroscopically, it tends to form polypoid masses and histologically, demonstrates irregularly distended, fused or branching tubules of various sizes sometimes with intraluminal mucus and nuclear and inflammatory debris. Papillary

adenocarcinoma is another variant that, histologically, is characterized by epithelial projections supported by a central fibrovascular core.

Mucinous adenocarcinoma is defined as a gastric adenocarcinoma with a substantial amount of extracellular mucin (≥ 50% of tumour volume) (Figure 6C). The neoplastic cells can form glandular structures or irregular cell clusters, with occasional scattered signet ring cells floating in the extracellular mucin lakes.

Signet ring cell adenocarcinoma of the stomach is characterized by diffuse infiltration of signet ring tumour cells in the gastric wall. Histologically, this lesion consists of isolated or small groups of malignant cells containing intracytoplasmic mucin with eccentric nuclei (Fig. 6D), often accompanied by marked desmoplasia with an infiltrative pattern. In some cases, as in human GCs, the canine signet ring tumours appear to form a *linitis plastica*-type tumour by spreading intramurally, and not usually involving the mucosa (unpublished observations).

The 2010 WHO classification of human GCs includes another histological variant termed 'other poorly-cohesive carcinomas'. These are often composed of a mixture of non-signet ring cells, morphologically resembling histiocytes, lymphocytes, and plasma cells. Those tumour cells can form irregular microtrebaculae or lace-like abortive glands, often accompanied by marked desmoplasia in the gastric wall and with a grossly depressed or ulcerated surface. Although the WHO classification for domestic animals (Head, 2003) does not recognize this specific histological type, we have observed some canine gastric neoplastic lesions that fit this description (Figure 6E).

In addition to the above major histological patterns of gastric cancers, the 2010 WHO human classification recognizes other variants, based on the presence or absence of poorly differentiated components, such as the mixed carcinoma. This entity consists of a well differentiated adenocarcinoma mixed with < 50% of poorly differentiated components. Equivalent tumours are recognized in the dog (unpublished observations).

Other uncommon histological variants of GC are rare in the dog. There is one report of neoplasia displaying features consistent with primary gastric choriocarcinoma in a 9-year-old, male Poodle (Poutahidis *et al.*, 2008). The microscopic examination of the area of pyloric thickening revealed extensive transmural infiltration by a highly malignant neoplasm with two distinct components: signet ring cells with eccentrically placed nuclei and clear cytoplasm; and highly pleomorphic cells plus bizarre multinucleate giant cells resembling, respectively, cytotrophoblasts and syncytiotrophoblasts (Poutahidis *et al.*, 2008).

Carcinoids are also rare neoplasms (Brown *et al.*, 2007) arising from neuroendocrine cells that share particular gross, histological, ultrastructural, histochemical and

immunohistochemical features. The first case of this type of lesion arising in the stomach of a 13-year-old female whippet was reported by Albers et al. (Albers *et al.*, 1998). Round to polyhedral neoplastic cells were observed as individual cells, small nests, short palisading cords or as acini, along the mucosa and infiltrating the antral stomach wall. The neoplastic cells were positive by Grimelius stain (argyrophilic) and immunohistochemically they expressed chromogranin, low and high molecular weight cytokeratins, neuron-specific enolase (NSE) and synaptophysin. The presence of intracytoplasmic neurosecretory granules was further confirmed by electron microscopy.

Qvigstad *et al.* (2008) proposed that the Norwegian Lundehund has an increased risk of developing chronic inflammation of the fundic mucosa, leading to atrophy and probably hypoacidity, and that these changes may progress to development of GCs, most likely of neuroendocrine origin (enterochromaffin-like cells) (Qvigstad *et al.*, 2008).

Gastrinoma is a rare malignant neuroendocrine neoplasia that results in autonomous gastrin secretion (Gal *et al.*, 2011). However, in contrast to the predominant origin of human gastrinomas from the gastric antrum and duodenum (Pipeleers-Marichal *et al.*, 1990), canine gastrinomas are supposed to arise from non-beta pancreatic islet cells (Simpson and Dykes, 1997). This neoplasm stimulates hypersecretion of gastric acid, resulting in severe gastric and duodenal ulceration and gastric mucosal hypertrophy, and also decreases the tone of the pyloric sphincter, allowing duodenal-gastric reflux of bile.

11.3 - Application of Molecular Pathology in Canine Gastric Carcinoma

In human medicine, the understanding of the molecular events and pathways in GC have led to advances in prevention, early diagnosis, tumour classification and therapeutic intervention. An accumulation of genetic and molecular abnormalities occurring during gastric carcinogenesis has been described in detail, including activation of oncogenes, overexpression of growth factors/receptors, inactivation of tumour suppression genes, involvement of DNA repair genes and cell adhesion molecules (Yasui *et al.*, 2006), loss of heterogeneity and point mutations of tumour suppressor genes, and silencing of tumour suppressors by CpG island methylation (Kitaura *et al.*, 1999).

In veterinary pathology the first steps towards molecular investigations are being taken. The reduced sampling and the difficulty in validating and optimizing the 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, since strong similarities have been observed between both species with regard to the clinical presentation and histopathological features of GC.

The Sialyl Lewis x–modified core 2 branched O-glycans (C2-O-sLex) on human leukocytes mediate much higher-affinity adhesion to selectins on activated vascular endothelium than does sialyl Lewis x on other structures. Janke *et al.* (2010) tested 16 canine gastric carcinomas and found an overexpression of C2-O-sLe(x) in 56% of the tumours, when compared with normal gastric mucosa. The authors hypothesized that these carbohydrates are highly expressed in more malignant types of canine gastric carcinomas; promote adhesion to selectins, attachment of cancer cells to the vascular endothelium and therefore, metastasis and a poorer prognosis .

The type I growth factor receptor family consists of the prototype EGFR and the related members, c-erbB-2, c-erbB-3 and c-erbB-4 (also known as HER-1, HER-2, HER-3 and HER-4, respectively). The membrane receptors that have a role in function have been shown to be important in the development of neoplasia (Wiseman *et al.*, 2005). The molecule HER-3 differs from the other members of this family because it has little or no tyrosine kinase activity. In man, blockade of HER-3 activity decreased downstream signalling of the phosphoinositide 3-kinase/protein kinase B pathway and the cell cycle, resulting in the death of tumour cells (Kunii *et al.*, 2008). Previous investigations have suggested a close relationship between cytoplasmic/membrane HER-3 expression and tumour prognosis for human GC (Hayashi *et al.*, 2008). Doster and colleagues (2011) determined CDX2 and HER-3 expression in five gastric mucinous adenocarcinomas. In all cases, strong cytoplasmic HER-3 labelling was recorded in > 10% of tumour cells and most nuclei of the neoplastic cells showed strong CDX2 expression. However, in this study, CDX2 and HER-3 expression could not be correlated with prognosis due to the small population size and the lack of a complete clinical history and post-surgical follow-up (Doster *et al.*, 2011).

A recent study in dogs focused on others members of this family, EGFR and its analogue HER-2, and KRAS, which is a small protein encoded by the *KRAS* gene that mediates the transduction of signals between EGFR receptors and the nucleus. In human oncology it has been shown that the *KRAS* gene can suffer mutations, most of which involve codons 12, 13 and 61 (exons 2 and 3) identified as a predictor of resistance to anti-EGFR drugs (Jimeno *et al.*, 2008). Terragni *et al.* (2014), evaluated EGFR/HER-2 immunoexpression and *KRAS* mutational status retrospectively in five adenomas and 14 carcinomas (five intestinal-type and nine diffuse-type carcinomas). Overexpression of EGFR and HER-2 was 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 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 man and leads to substitution of a glycine by an arginine, thus leading to a geometric alteration of the protein (Terragni *et al.*, 2014).

This results in a lack of GTP hydrolysis which keeps *KRAS* in a permanently activated state (Eberhard *et al.*, 2005).

Genetic and/or epigenetic alterations in 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 in metastasis (Carneiro *et al.*, 2012). In man, *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. We are currently evaluating the potential role of E-cadherin (E-cad) and its encoding-gene (*CDH1*) in canine GCs. Our preliminary results show that the majority of canine CGs have abnormal E-cad expression in comparison with the normal gastric mucosa. The different patterns of E-cad immunoexpression observed in canine GCs are similar to those already described in human GCs and the existence of *CDH1* somatic alterations in such lesions may provide useful information for the clinical management and prognosis of these animals (Amorim *et al.*, 2014a).

In another investigation, the expression of gastrin was investigated immunohistochemically in gastric biopsy samples from 64 dogs with gastric carcinoma. Only 8% of the canine carcinomas demonstrated expression of gastrin which is considered less common than in man. The authors concluded that this may not be a reliable prognostic marker in the dog (Seim-Wikse *et al.*, 2014).

12 – Conclusions

This review has examined the spectrum and classification of lesions of the canine stomach and compared these with the equivalent human disorders. The use of standardized reporting systems for grading the histological changes in canine gastric biopsy samples is required in order to standardize the interpretation of lesions by veterinary pathologists. Some such schemes, such as that proposed by the WSAVA GI Standardization Group for gastric inflammation (Day *et al.*, 2008) have been introduced, but more refined classification schemes are required for canine gastric cancer in which histological and immunohistochemical features, together with molecular pathogenesis, might be considered. Such schemes should not be developed in isolation and must be related to the biological behaviour and response to therapy of the tumours considered.

Canine gastric lesions often show great histological similarities to their human

counterparts. Head *et al.* reinforce that the WHO histological classification of human gastric tumours should be followed as far as possible (Head and Ring, 2003); however, many canine gastric carcinomas may still be classified erroneously because: (1) endoscopic biopsies are superficial and fail to obtain the representative features of the lesion; (2) veterinary pathologists are unfamiliar with the human classification scheme; and (3) immunohistochemical studies are not performed.

The application of molecular biology techniques to this field of veterinary pathology will lead to greater understanding and new diagnostic approaches; however, many challenges still remain in canine gastric pathology.

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Table 1 – Causes of gastritis or gastric ulceration in dogs

Adverse reactions to food
Food allergy (hypersensitivity)
Food intolerance
Dietary indiscretation
Chemicals
Foreign bodies
Garbage toxicosis
Gluttony
Heavy metal toxicosis
Plants
Drug administration
Corticosteroids
Non-steroidal antiinflammatory agents
Infectious agents
Fungi (*Physaloptera*)
Parasites
Spiral bacteria (*Helicobacter* spp.)
Neoplasia
Gastrinoma
Mastocytosis
Primary gastric neoplasia
Reduced gastric blood flow
Disseminated intravascular coagulopathy
Neurological disorders
Sepsis
Shock
Uremia
Systemic disease
Hypoadrenocorticism
Kidney disease
Liver disease
Pancreatitis
Reflux gastritis
Inflammatory bowel disease (IBD)
Idiopathic gastritis

Table 2 – Comparison between the different gastric carcinoma classification systems

WHO classification for alimentary tumours of domestic animals **Head et al. (2003)**	**WHO Human Classification (2010)**	**Human classification by Lauren (1965)**
Tubular adenocarcinoma Papillary adenocarcinoma Mucinous adenocarcinoma	Tubular adenocarcinoma Papillary adenocarcinoma Mucinous adenocarcinoma	Intestinal type
Signet-ring cell carcinoma	Signet-ring cell carcinoma Other poorly cohesive carcinoma	Diffuse type
	Mixed carcinoma	Indeterminate type
	Adenosquamous carcinoma	
Squamous cell carcinoma	Squamous cell carcinoma	
	Hepatoid adenocarcinoma	
	Carcinoma with lymphoid stroma	
	Choriocarcinoma	
	Carcinosarcoma	
	Parietal cell carcinoma	
	Malignant rhabdoid tumor	
	Mucoepidermoid carcinoma	
	Paneth cell carcinoma	
Undifferentiated carcinoma	Undifferentiated carcinoma	
	Mixed adeno-neuroendocrine carcinoma	
	Endodermal sinus tumour	
	Embryonal carcinoma	
	Pure gastric yolk sac tumor	
	Oncocytic adenocarcinoma	

Figures

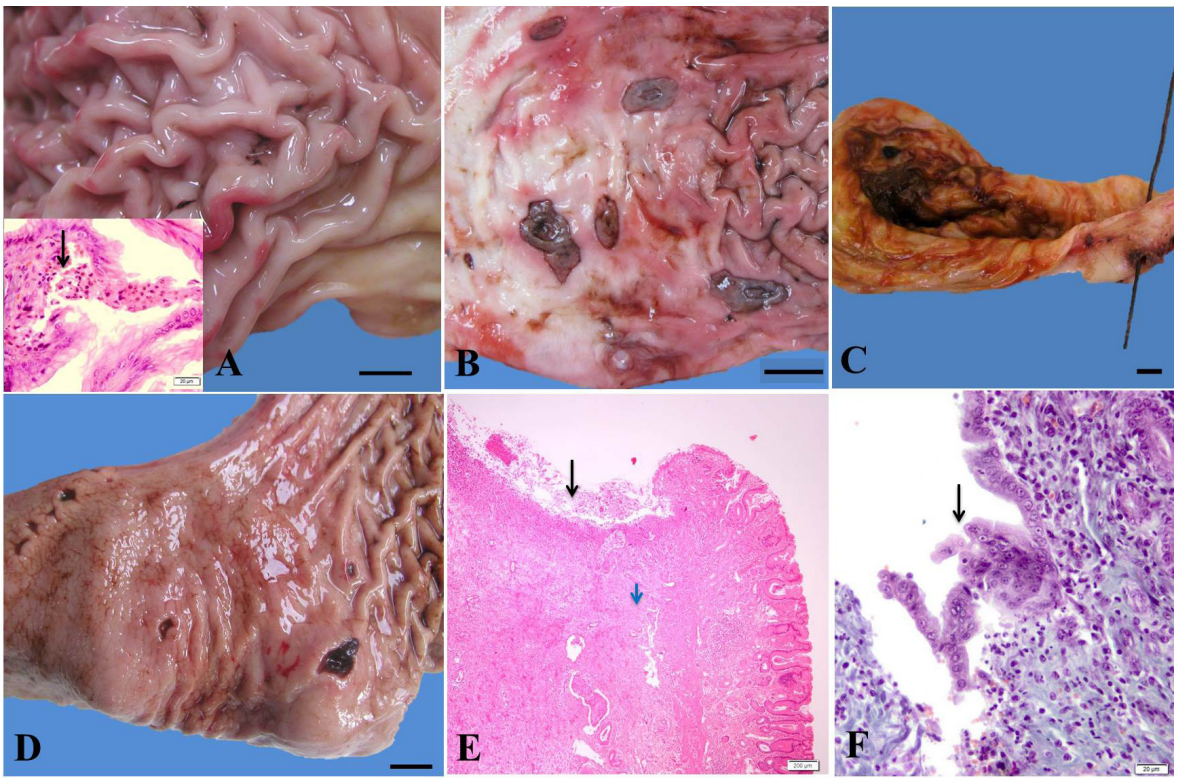


Figure 1 – Morphological features of canine gastric erosion and ulceration. A) Acute multifocal gastric erosions in a 5 month-old mixed breed puppy. Multiple, pinpoint gastric ulcers with small blood clots on the mucosal surface of the body region (arrows). Bar=1cm; the inset shows focal necrosis of overlying epithelium, microhemorrhages and infiltration of neutrophilic leukocytes in the superficial *lamina propria*. Hematoxylin-eosin staining (HE). Bar=20µm; B) Gross image of multiple acute antral ulcers. Bar=1cm; C) A solitary, large and perforated ulcer (trajectory shown with probe) with haemorrhagic infiltration of the wound margins; digested blood is also present in the gastric content. Bar=1cm; D) Multiple and large gastric ulcers with irregular white to grey and dense elevated borders, filled with blood clots in the corpus and pyloric regions. Bar=1cm; E) Photomicrograph showing a chronic ulcer consisting of a gastric wall defect extending to the submucosa and muscularis mucosa (black arrow) and delimited by a granulation tissue of variable thickness and maturity (blue arrow). HE. Bar=200 µm; F) Chronic ulcer with marked granulation tissue proliferation infiltrated by mononuclear cells and neutrophils, and signs of epithelial cell regeneration in one of the edges of the lesion (arrow). Masson trichrome staining (MT). Bar=20µm.

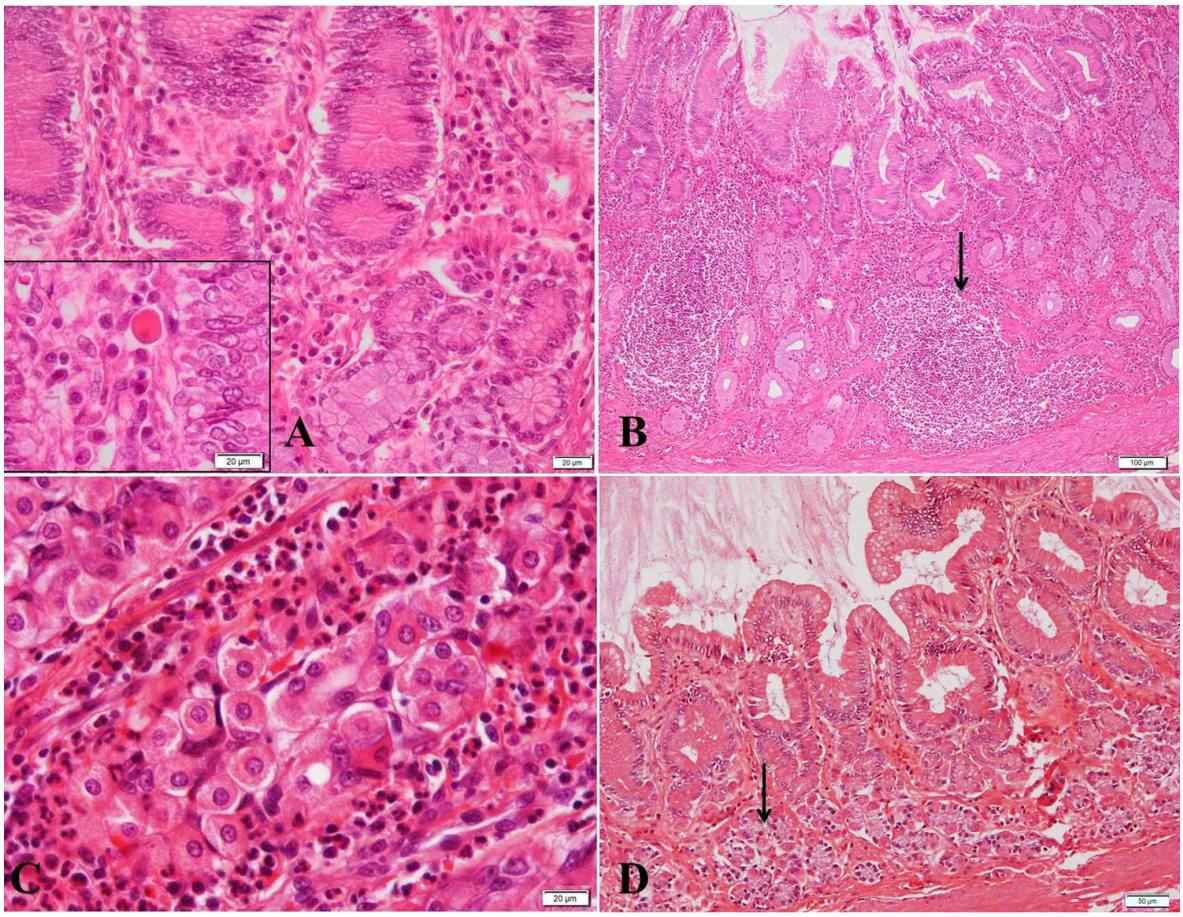


Figure 2 - Morphological features of canine chronic gastritis. A) Antral lymphoplasmacytic gastritis with moderate foveolar hyperplasia and Mott cell infiltration into the *lamina propria* (inset, bar=20µm). HE. Bar=50µm; B) Gastric body region showing lymphoplasmacytic gastritis with concomitant lymphoid follicle hyperplasia. HE. Bar=100µm; C) Moderate eosinophilic gastritis of the gastric body. HE. Bar=100µm; D) Chronic atrophic gastritis characterized by mononuclear infiltration, glandular atrophy (arrow) and fibrosis of *lamina propria*. HE. Bar=50µm.

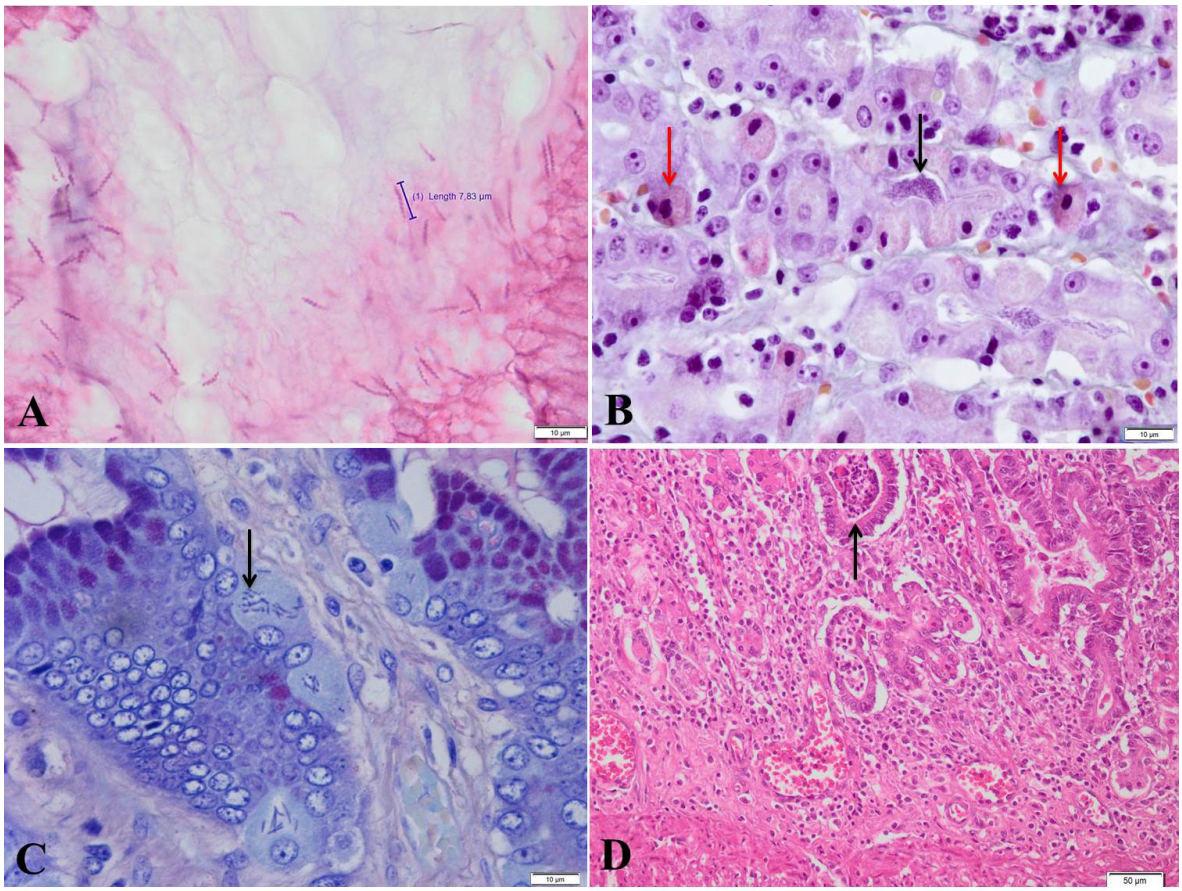


Figure 3 - *Helicobacter spp*. in canine stomach. A) NHPH colonizes the superficial epithelium of the pyloric region in a dog. H&E. Bar=10µm; B) NHPH within the gastric glands of the body region (black arrow); note the marked degeneration of the parietal cells (red arrows). MT. Bar=10µm; C) Intracellular localization of NHPH organisms (parietal cells) (arrow) Modified Giemsa staining. Bar=10µm; D) Chronic-active *Helicobacter* spp. related gastritis of the fundic region. Severe inflammatory infiltration of macrophages, plasma cells, lymphocytes and neutrophils within the lamina propria with congestion, degeneration of gastric glands and “pit abscess” (arrow). Bar=50µm (HE).

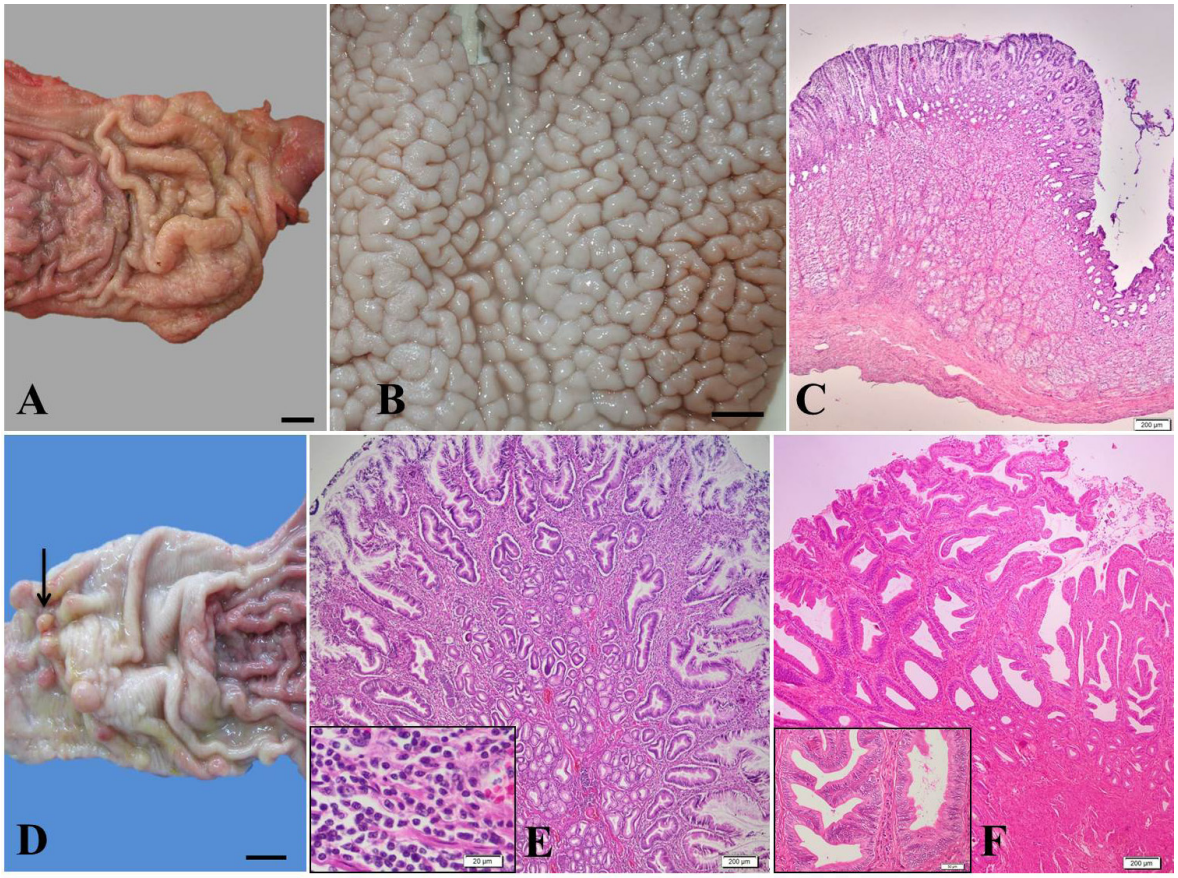


Figure 4 - Gross and histological findings of canine hypertrophic gastritis and gastric polyps. A). Marked thickening of the gastric mucosa in the pyloric region. Bar=1cm; B) The body region showing diffuse hypertrophic gastritis with cerebriform appearance of the rugal folds (formalin fixed tissue). Bar=1cm; C) Marked foveolar and gastric gland hyperplasia and dilation, and mucus (pseudopyloric) metaplasia of the fundic glands. Bar=200µm. Inset shows the superficial *lamina propria* with severe inflammatory infiltration by mononuclear cells (Bar=50 µm) (HE staining); D) Multiple inflammatory polyps in the pyloric region (arrow). Bar=1cm; E) Inflammatory gastric polyp with moderate foveolar hyperplasia and marked infiltration of the *lamina propria* with lymphocytes and plasma cells; inset shows a detail of the infiltrate. Bar=200µm, respectively 20µm (HE staining); F) Adenomatous polyp composed of tubular and/or villous structures lined by dysplastic epithelium; inset shows the hyperplastic epithelium with tubulopapillary architecture. Bar=200µm, respectively 50µm (HE staining).

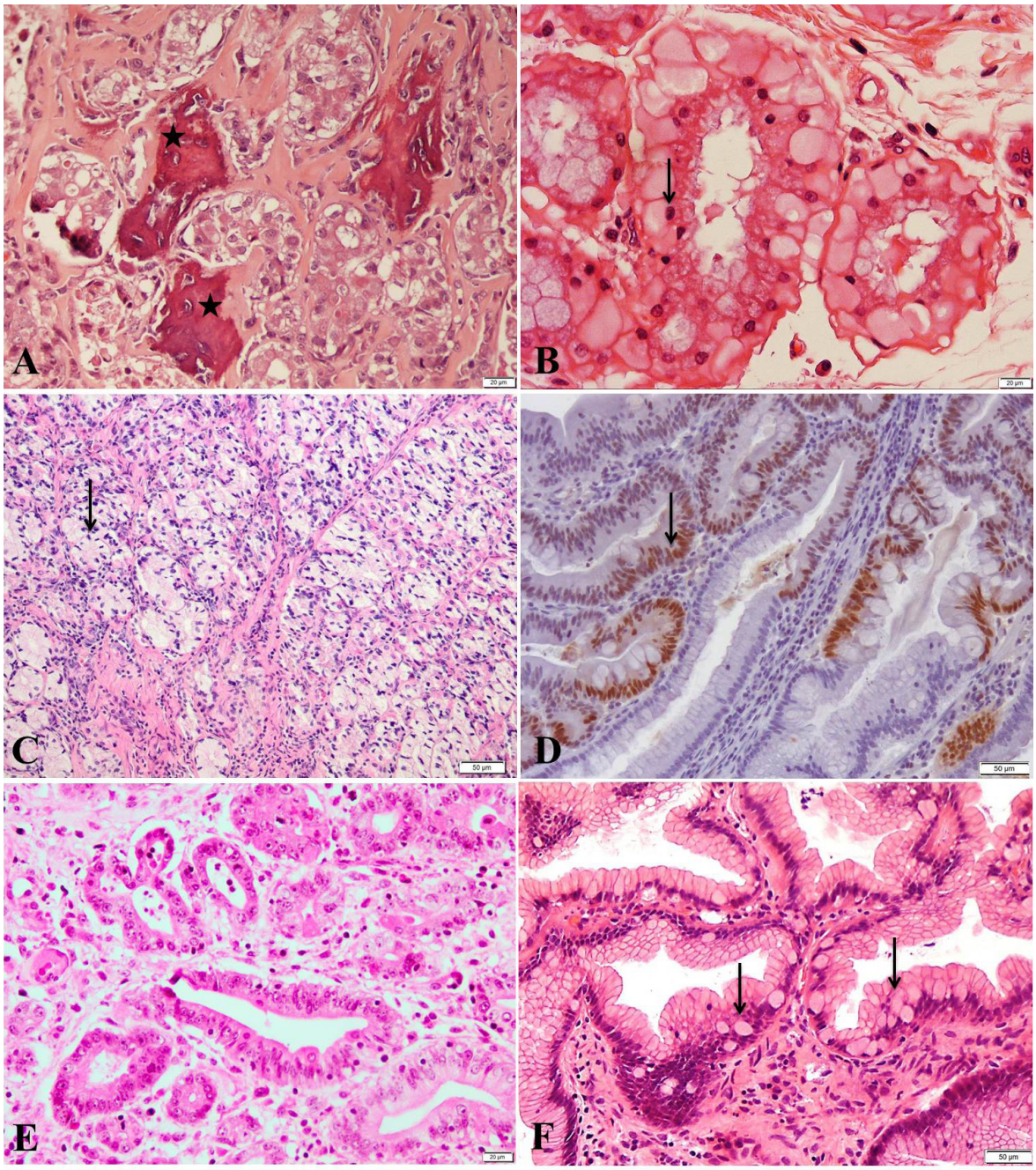


Figure 5 - Histological aspects of different metaplastic and dysplastic changes of canine gastric mucosa. A) Osseous metaplasia (heterotopic bone formation) consisting of osteoid and fully mature bone associated with a gastric carcinoma (black asterisks). H&E. Bar=20µm; B) Glands from the cardia showing glassy cell change, characterized by cells with a eosinophilic cytoplasm with ground-glass appearance and luminal, small and eccentric nuclei. HE. Bar=20µm; C) The fundic region revealing diffuse and marked replacement of parietal and chief cells by mucous cells (mucous metaplasia) (arrow). H&E. Bar=50µm; D) CDX2 immunohistochemical reactivity of Goblet

cells (arrow) in a gastric tubulopapillary adenocarcinoma. Immunoperoxidase-diaminobenzidine stain with Mayer's haematoxylin counterstain. Bar=50μm; E) High-grade epithelial dysplasia of fundic mucosa characterized by highly distorted glandular architecture, irregular and dilated glands covered by a stratified epithelium with crowded and pleomorphic nuclei. H&E. Bar=20μm; F) The foveolar epithelium from pyloric region presenting diffuse globoid dysplasia. Cells have an abundant and vacuolated cytoplasm, with excessive amounts of mucin, which compress the eccentric and hyperchromatic nuclei, resembling signet ring-cells (arrows). HE. Bar=50μm.

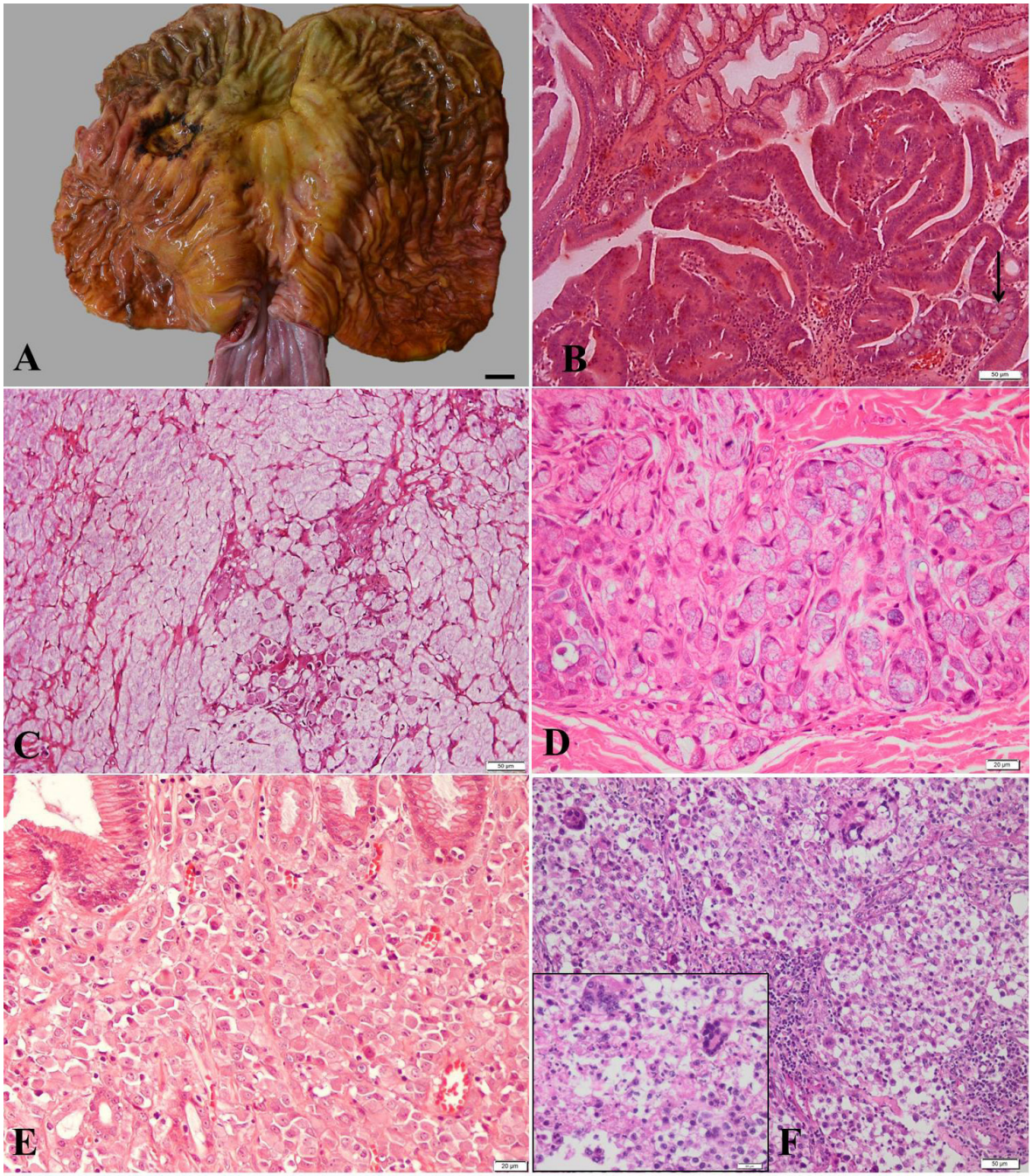


Figure 6 - Morphological appearances of gastric epithelial neoplasia in dogs. A) Diffuse thickening and ulceration of the gastric wall located in the lesser curvature. Bar=1cm. Photomicrographs revealing several histological variants of canine gastric carcinomas: B) Tubulopapillary type. HE. Bar=50µm; C) Mucinous type. HE. Bar=50µm; D) Poorly cohesive carcinoma. HE. Bar=20µm; E) Signet ring cell carcinoma. HE. Bar=20µm; F) Primary gastric choriocarcinoma. Bar=50µm. Inset shows bizarre epithelial multinucleate giant cells. Bar=50µm. HE staining.

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-Aim and Objectives

General aim:

To characterize the epidemiological, pathological and molecular features of the spontaneous gastric infection with different NHPH in canine stomach and to evaluate the relevance of particular glycan structures in *Helicobacter spp.* adhesion to canine gastric mucosa.

Specific aims:

Paper I

- To examine the spectrum, classification, pathophysiology, macroscopic and histopathological findings of the most common canine gastric lesions and compare these with the equivalent human disorders.

Paper II

In humans, the normal gastric mucosa glycosylation profile is characterized by the expression of neutral glycans, including the expression of type 1 Lewis antigens, Lea and Leb, in the superficial foveolar epithelium and type 2 Lewis antigens, Le^x and Le^y, in the glands. Sialylated antigens are rarely found in health conditions. In chapter 2, we intend to: will characterize the glycosylation profile of the normal canine gastric mucosa.

- To characterize the glycosylation profile of canine gastric mucosa, focusing on the expression of the histo-blood group antigens, in order to understand their relevance in NHPH adhesion.

- To evaluate the adhesion ability of different *Helicobacter* species to the canine gastric mucosa. The *in vitro* binding capacity of FITC-labelled *H. pylori* and NHPH to the canine gastric mucosa will be assessed in cases representative of the canine glycosylation pattern.

Paper III

Several works have discussed the prevalence of *Helicobacter spp.* in dogs, but few determine the specific species present in the canine stomach. The identification of NHPH at the species level has become possible through the use of PCR, allowing the determination of the prevalence of these various species in human and canine stomach biopsies. The accurate identification of Helicobacter spp. is essential in order to determine the prevalence and clinical significance of all taxa. In the chapter 3 we intend:

- To determine the prevalence of *Helicobacter spp.* in the canine stomach;

- To determine the distribution of *Helicobacter spp.* in the different portions of canine gastric mucosa and correlate it with possible histopathological alterations;

- To determine the specific *Helicobacter* species involved in canine gastric infection.

Paper IV

- To characterise the molecular features of particular canine gastric lesions.

In humans, and in contrast to the situation verified in the colon, most gastric polyps are considered non-neoplastic lesions. In dogs, gastric polyps are uncommon lesions thus, the molecular properties and biological significance of these canine lesions remains a poorly explored area. In chapter 4, we intended to analyse immunohistochemically a series of spontaneous canine gastric polyps regarding the presence of *Helicobacter spp.*, cellular proliferative activity, potential phenotypic alterations and COX-2 and p53 expression. Additionally, the expression of these molecules in normal gastric mucosa will be investigated to evaluate the significance of their loss or overexpression in the gastric lesions.

- ***Helicobacter spp.*** **adhesion to canine gastric mucosa**

CHAPTER 2

Paper II

A comparison of Helicobacter pylori and non-Helicobacter pylori Helicobacter spp. binding to canine gastric mucosa with defined gastric glycophenotype

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A comparison of *Helicobacter pylori* and non-*Helicobacter pylori Helicobacter* spp. Binding to Canine Gastric Mucosa with Defined Gastric Glycophenotype

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Keywords
Canine gastric mucosa, bacterial adhesion, *Helicobacter pylori*, histo blood group antigens, non-*Helicobacter pylori* helicobacters.

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Abstract

Background: The gastric mucosa of dogs is often colonized by non-*Helicobacter pylori* helicobacters (NHPH), while *H. pylori* is the predominant gastric *Helicobacter* species in humans. The colonization of the human gastric mucosa by *H. pylori* is highly dependent on the recognition of host glycan receptors. Our goal was to define the canine gastric mucosa glycophenotype and to evaluate the capacity of different gastric *Helicobacter* species to adhere to the canine gastric mucosa.

Materials and Methods: The glycosylation profile in body and antral compartments of the canine gastric mucosa, with focus on the expression of histo-blood group antigens was evaluated. The in vitro binding capacity of FITC-labeled *H. pylori* and NHPH to the canine gastric mucosa was assessed in cases representative of the canine glycosylation pattern.

Results: The canine gastric mucosa lacks expression of type 1 Lewis antigens and presents a broad expression of type 2 structures and A antigen, both in the surface and glandular epithelium. Regarding the canine antral mucosa, *H. heilmannii* s.s. presented the highest adhesion score whereas in the body region the SabA-positive *H. pylori* strain was the strain that adhered more.

Conclusions: The canine gastric mucosa showed a glycosylation profile different from the human gastric mucosa suggesting that alternative glycan receptors may be involved in *Helicobacter* spp. binding. *Helicobacter pylori* and NHPH strains differ in their ability to adhere to canine gastric mucosa. Among the NHPH, *H. heilmannii* s.s. presented the highest adhesion capacity in agreement with its reported colonization of the canine stomach.

The gastric mucosa of dogs is often colonized by helicobacters different from *Helicobacter pylori* (*H. pylori*). These non-*H. pylori* helicobacters (NHPH) are present in 67–86% of clinically healthy dogs, in 61–100% of animals presenting chronic vomiting [1–5] and in about 100% of laboratory dogs and dogs from local shelters [6–8]. The predominant gastric *Helicobacter* species in dogs are *H. felis, H. bizzozeronii,* and *H. heilmannii* sensu stricto (s.s.), while *H. salomonis* is less often detected and the prevalence of *H. cynogastricus* and *H. baculiformis* has not yet been studied [9–12]. Mixed infections with different species can also occur [13]. The pathogenic significance of gastric NHPH in dogs is poorly understood and remains controversial; therefore veterinarians are facing the dilemma of either treating or ignoring spiral organisms observed in canine gastric biopsies. However, these NHPH are of zoonotic significance and have been associated with gastritis, peptic ulcers and mucosa associated lymphoid tissue lymphomas in human patients [11]. Dogs may constitute a source of

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infection for their owners, although the prevalence of these canine- and feline-associated helicobacters in humans is much lower than that of *H. pylori*. On the other hand, *H. pylori* has occasionally been identified in the canine stomach [14,15]. Gastric *Helicobacter* species have a host species preference, but may occasionally cross this host species barrier. The molecular mechanisms underlying this partial host-adaptation are not known, but may be related to differences in glycosylation profile of the gastric mucosa.

The gastrointestinal tract glycosylation profile determines the colonization capability of various infectious agents. Bacterial binding occurs through recognition of specific glycan receptors expressed by the host epithelial cells [16–18]. The biosynthesis of glycan chains is controlled by enzymatic activity of several glycosyltransferases, which are expressed in a cell, tissue and species-specific manner (for review see [19]).

The expression of the terminal Lewis glycan structures that are recognized as receptors for *H. pylori* in human gastric tissue depends on the enzymatic activity of specific fucosyltransferases and sialyltransferases [20] and are schematically represented in Fig. 1A, B. Two backbone structures are the precursors for the Lewis antigens biosynthesis: the type 1 Galβ1-3GlcNAc, and the type 2 Galβ1-4GlcNAc chains. Addition of a fucose to terminal galactose on type 1 structures leads to H-type 1 structure, which can be further modified with a fucose on the GlcNAc residue resulting in Lewis b (Le^b). Alternatively, the type 1 backbone may be fucosylated on the GlcNAc residue leading to Lewis a (Le^a) structure. Furthermore, the action of a sialyltransferase toward type 1 chains can lead to the biosynthesis of the sialyl-Le^a (sLe^a) antigen. The biosynthesis of type 2 based Lewis antigens depends on the addition of the same glycan units but with different linkages, originating the isomers Lewis x (Le^x), Lewis y (Le^y) and sialyl-Le^x (sLe^x) antigens (Fig. 1B) (as reviewed by [20]).

The *H. pylori* blood group antigen-binding adhesin (BabA) recognizes both H-type 1 and Le^b fucosylated antigens [16] expressed on the surface of the gastric mucosa of secretor individuals [21,22]. The secretor status is determined by the activity of the fucosyltransferase 2 enzyme (FUT2) [23] and the capacity to synthesize H-type 1 and Le^b antigens in body secretions. The secretor and Lewis status in humans are associated with the adhesion and infection of *H. pylori* strains expressing the BabA adhesin [21,24,25]. In addition, a second *H. pylori* adhesin, the sialic acid-binding adhesin (SabA) has been described, which recognizes the sialylated antigens sLe^a and sLe^x [17]. These sialylated structures are absent in normal human gastric mucosa but are induced upon *H. pylori* infection and gastric mucosa inflammation [17,26,27].

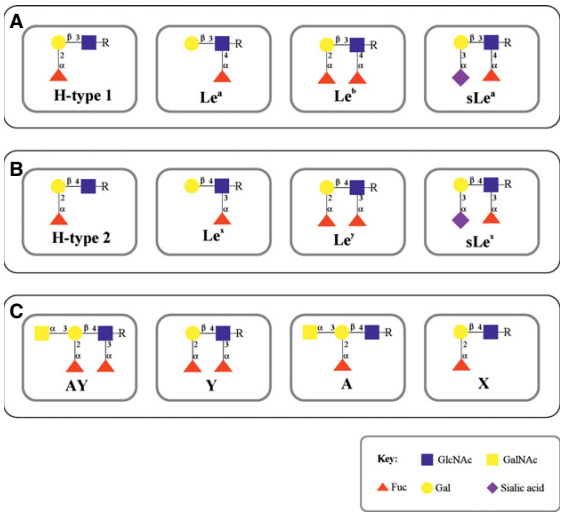


Figure 1 Schematic representation of Lewis antigens. Type 1 chains (panel *A*) are characterized by the Galβ1,3 linkage, while type 2 chains (panel *B*) display a Galβ1,4 linkage. (Panel *A*) Addition of a fucose to terminal galactose on type 1 structures leads to H-type 1 structure, which can be further modified with a fucose on the GlcNAc residue resulting in Le^b antigen. The type 1 backbone may be just fucosylated on the GlcNAc residue leading to Le^a antigen biosynthesis. The action of a sialyltransferase towards the type 1 chain can lead to the biosynthesis of the sLe^a antigen. (Panel *B*) the biosynthesis of type 2 based Lewis antigens depends on the addition of the same glycan units but with different linkages, originating the isomers Le^x, Le^y and sLe^x antigens. Addition of a fucose to terminal galactose on type 2 structures leads to H-type 2, which can be further modified with a fucose on the GlcNAc residue resulting in Ley antigen. The type 2 backbone may be just fucosylated on the GlcNAc residue leading to Le^x antigen biosynthesis. The action of a sialyltransferase toward the type 2 chain can lead to the biosynthesis of the sLex antigen. (Panel *C*) Schematic representation of canine secretory alloantiagens (CSA) polymorphisms (adapted from Oriol et al. [30]).

The canine secretory alloantigen alloantibody system (CSA) is closely related to the human ABH-Le system being equivalent to the major human ABO exocrine glycophenotype. The CSA was first described in 1966 by Zweibaum and colleagues [28]. The genetic polymorphisms of the CSA were later analysed [29] and the structure of canine polymorphic antigens was further characterized in four phenotypes: A, X, Y, and AY [30] (Fig. 1C). However, limited information is available about the glycosylation profile of the canine gastric mucosa.

Here we describe, for the first time, the glycophenotype observed in the canine gastric mucosa, with focus on the expression of Lewis glycan antigens. Furthermore,

the binding capacity of *H. pylori* and NHPH to canine gastric mucosa was evaluated. This study provides valuable information about the canine gastric glycosylation profile and about the Helicobacter capacity to adhere to the canine gastric mucosa, contributing to the understanding of the host colonization spectrum of the different *Helicobacter* species.

Materials and Methods

Tissue Samples and Histology

Gastric samples were obtained during necropsy procedures from dogs that died from non-infectious causes (eight representative sections of the body and six of the antrum of the stomach).

Tissues were fixed in 10% buffered formalin and paraffin-embedded. Serial sections 3 μm thick were made, one being stained with haematoxylin and eosin (HE) for histopathology and the others were used for the histochemical and immunohistochemical studies.

Sections stained with HE were examined by three pathologists and were considered as normal gastric tissues, according to the criteria proposed by Prachasilpchain et al. [31]. Additionally, the presence of *Helicobacter* spp. was excluded based on the negative results of modified Giemsa stain and of anti-*H. pylori* immunohistochemistry, using a polyclonal antibody (RBK012; Zytomed, Berlin, Germany), which shows immunoreactivity with a wide range of bacteria belonging to the *Helicobacter* genus.

Immunohistochemistry

For the immunohistochemical study, sections were deparaffinized, hydrated and antigen retrieval was performed in a pressure cooker in 10 mmol/L sodium citrate buffer, pH 6.0, for 2 minutes. Slides were cooled for 10 minutes at room temperature and rinsed twice in triphosphate buffered saline (TBS) for 5 minutes. The Novolink™ Max-Polymer detection system (Novocastra, Newcastle, UK) was used for visualization, according to the manufacturer's instructions. After blocking endogenous peroxidase with 3% hydrogen peroxide in methanol for 10 minutes, sections were incubated, overnight at 4 °C, with the monoclonal antibodies specific for the carbohydrate antigens (Table 1). Sections were rinsed with TBS between each step of the procedure. Color was developed for up to 7 minutes at room temperature with 3,3′-diamino-benzidine (DAB; Sigma, St. Louis, MO, USA) and sections were then lightly counterstained with haematoxylin, dehydrated, and mounted.

Table 1 Antibodies used in immunohistochemistry

Monoclonal antibody	Antigen	Supplier/ References	Dilution
CA3F4	Le^a	Young et al. [55]	1 : 5
BG6	Le^b	Signet© (Dedham, MA, USA)	1 : 50
CA19-9	Sialyl-Le^a	Santa Cruz© (Santa Cruz, CA, USA)	1 : 500
SH1	Le^x	Fukushi et al. [56]	1 : 5
KM93	Sialyl-Le^x	Calbiochem© (Darmstadt, Germany)	1 : 60
AH6	Le^y	Abe et al. [57]	1 : 10

Sections of human gastric mucosa with intestinal metaplasia were obtained from the pathology department archive of Hospital Santo Antonio – Porto and were used as positive controls. Negative controls were performed by replacing the primary antibody with an antibody of the same immunoglobulin isotype.

Lectin Staining

The biotinylated lectin *Dolichus biflorus* agglutinin (DBA), for detection of terminal alpha linked GalNAc, and the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit) solution were purchased from Vector Laboratories (Burlingame, CA, USA). After deparaffination and rehydration, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 minutes at room temperature. After washing twice with phosphate buffered saline (PBS) for 5 minutes, sections were incubated with 10% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature and then in the biotinylated lectin solution (1 : 100 in PBS) for 1 hour, at room temperature. The slides were subsequently washed in PBS and immersed in ABC solution for 30 minutes at room temperature, according to manufacturer's recommendations. After washing twice with PBS for 5 minutes, slides were immersed in a freshly prepared solution of DAB containing 0.02% hydrogen peroxide for 7 minutes at room temperature to visualize the lectin binding sites, counterstained with haematoxylin, dehydrated and mounted. Sections of canine normal intestine were used as positive controls. Negative controls were performed by replacing the lectin by PBS.

Evaluation of Gastric Tissue Glycosylation Profile

A microscopic evaluation was performed by analyzing the entire section of the gastric tissue. Positive immunoreactivity was recorded as a distinct brown labeling of cytoplasm and/or membrane of epithelial cells. Extracellular staining of the superficial mucus was

excluded from our evaluation. The immunostaining was scored according to the intensity (0, absent; +, weakly positive; + +, moderately positive; or + + +, intense stain) and distribution (sparse, focal, multifocal, and generalized).

Bacterial Strains

The *H. pylori* strains 17875/Leb and 17875babA1::kan babA2::cam (17875*babA1A2*) were obtained from the Department of Medical Biochemistry and Biophysics, Umeå University, Sweden [17]. These model strains were selected based on their capacity to adhere to human gastric mucosa tissue sections. The 17875/Leb strain is a spontaneous mutant which expresses both BabA and SabA adhesins, however, does not show sialylated dependent binding. The 17875*babA1A2* is a BabA adhesin mutant strain which expresses a functional SabA adhesin [17].

Helicobacter pylori were grown in Pylori agar (BioMérieux, Marcy l'Étoile, France) at 37 °C under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2; 37 °C). For strain *babA1A2*-mutant, culture media included also 20 mg/L Chloramphenicol (Sigma) and 25 mg/L Kanamycin (Sigma).

The NHPH strains were provided by the Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University. The *H. felis* CS1, *H. salomonis* R1053 and *H. bizzozeronii* R1051 strains were cultured biphasically on Brain Haert Infusion agar plates (BHI; Oxoid, Basingstoke, UK) supplemented with 10% horse blood, 20 μg/mL amphotericin B (Fungizone; Bristol-Myers Squibb, Eppernon, France), Vitox supplement (Oxoid), and Campylobacter selective supplement (Skirrow, containing 10 μg/mL vancomycin, 5 μg/mL trimethoprim lactate, and 2.5 U/mL; Oxoid). The *H. heilmannii* strain ASB1.4 was cultivated biphasically on *Brucella* agar plates (Oxoid) supplemented with 20% (v/v) fetal calf serum (HyClone, Logan, UT), 5 mg/L amphothericin, Skirrow and Vitox supplements (Oxoid), and 0.05% HCl (pH 5). All strains were incubated under microaerobic condition.

In vitro Binding Assay of *Helicobacter pylori* and NHPH to Canine Gastric Mucosa

For the in vitro Helicobacter binding assay we selected three representative cases (n = 3) of the general canine glycosylation pattern, belonging to three different dogs, in which body and antrum samples were available.

The different Helicobacter strains were labeled with fluorescein isothiocyanate (FITC) and concentration adjusted as previously described [32]. Adhesion assays were performed as previously described [22,32] with the following modifications. Briefly, paraffin embedded canine gastric sections were deparaffinized and rehydrated followed by overnight incubation at 4 °C and 1 hour at room temperature, with blocking buffer: 1% BSA in PBS containing 0.05% Tween 20. BSA was previously submitted to Periodate oxidation to destroy competitive carbohydrate receptors for Helicobacter binding [33]. The FITC-labeled bacterial suspension was diluted 5-fold in blocking buffer and 100 μL was used to incubate each section for 2 hours at room temperature. Slides were subsequently washed five times with PBS containing 0.05–0.1% Tween 20 and stained with DAPI. Evaluation was estimated by the number of adhered bacteria under 100× magnification, and analyzing at least five different fields per case and quantified using the ImageJ software (NIH, Bethesda, MD, USA).

Statistical Analysis

The Student's *t*-test was used to compare the differences between the mean adhesion values of both stomach regions for the same NHPH strain. The significance of differences between mean values of each Helicobacter strain was assessed using a one-way ANOVA (GraphPad Prism, GraphPad Software, Inc., La Jolla, CA, USA). p values < 0.05 were considered as statistically significant.

Results

Expression of Type 1 Lewis Antigens in Dog Gastric Mucosa

There was no Le^a antigen expression in the body region of the stomach from seven of the eight canine cases studied (Fig. 2A, Table 2). Only in one case, a very weak and sparse Le^a expression was detected in few superficial foveolar epithelial cells. In the antral region of the stomach, there was no Le^a antigen expression in all six cases studied (Fig. 2B, Table 2). $Lewis^b$ and sLe^a expression were absent in both the body and antrum of the stomach (Fig. 2D,E,G,H, Table 2).

Expression of Type 2 Lewis Antigens in Dog Gastric Mucosa

In the body of the stomach, expression of Le^x was seen in five of the eight canine cases (Table 2). The staining intensity varied from moderate to strong, always limited to the superficial foveolar epithelium (Fig. 2J). In all positive cases, the staining was multifocal and

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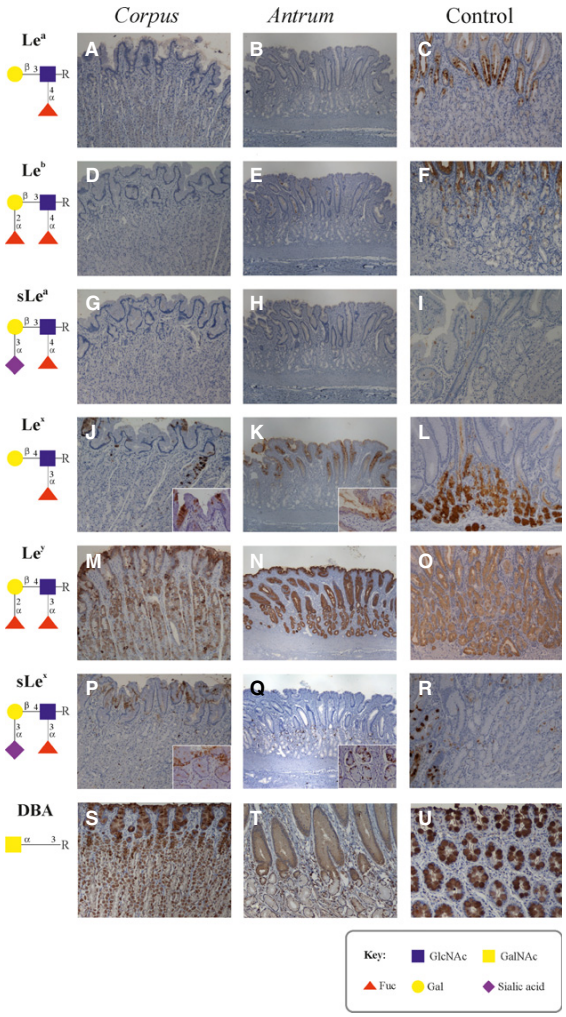


Figure 2 Expression of Lewis antigens and *Dolichus biflorus* agglutinin (DBA) lectin staining in gastric mucosa: (*A*) and (*B*) Normal canine gastric mucosa, IHC Le^a, 100× and 40× respectively. There was no Le^a antigen expression in both body and antrum. (*D*) and (*E*) Normal canine gastric mucosa, IHC Le^b, 100× and 40× respectively. There was no Le^b antigen expression in both body and antrum. (*G*) and (*H*) Normal canine gastric mucosa, IHC sLe^a, 100× and 40×, respectively. There was no sLe^a antigen expression in both body and antrum. (*J*) Normal canine gastric body, IHC Le^x, 100×. Multifocal and moderate cytoplasm expression of Le^x in the superficial foveolar epithelium. (*K*) Normal canine gastric antrum, IHC Le^x, 40×. Generalized and moderate staining of the superficial foveolar epithelium. (*M*) Normal canine gastric body, IHC Le^y, 100×. Generalized and intense immunostaining of superficial foveolar epithelium andparietal cells. (*N*) Normal canine gastric antrum, IHC Le^y, 40×. Intense and generalized expression of Le^y in foveolar epithelium and gastric pits. (*P*) Normal canine gastric body, IHC sLe^x, 100×. Multifocal and intense immunostaining of superficial foveolar epithelium. (*Q*) Normal canine gastric antrum, IHC sLe^x, 40×. Sparse and weak immunostaining of the foveloar superficial epithelial cells and in cells of the pyloric glands. (*C*), (*F*), (*I*), (*L*), (*O*), (*R*) Expression of Lewis antigens in human gastric mucosa with intestinal metaplasia used as positive controls, 100×. (*S*) Normal canine gastric body, *Dolichus biflorus* agglutinin (DBA), 100×. Generalized and intense staining of superficial foveolar epithelium and parietal cells. (*T*) Normal canine gastric antrum, DBA, 100×. Intense and generalized staining of foveolar epithelium and gastric pits, contrasting with lesser intensity in deeper pyloric glands. (*U*) DBA staining in normal canine intestinal mucosa used as positive control, DBA, 200×.

restricted to the cytoplasm. In the antrum of the stomach, Le^x expression was found in all cases. The staining intensity varied from weak to strong and was limited to the superficial foveolar epithelium. The pattern of staining was multifocal in four cases and generalized in the other two cases (Fig. 2K, Table 2).

All cases evaluated presented Le^y in the body and antrum. In the body, the level of immunostaining was weak in one case and intense in seven cases. The staining included superficial foveolar epithelium and parietal cells (Fig. 2M). The distribution of the staining was multifocal in four cases and generalized in the other four. The subcellular staining was mainly localized in the cytoplasm of the cells. In the antrum, the level of Le^y immunostaining varied from moderate to intense, presenting stronger labeling in the cells of the foveolar epithelium and gastric pits. The pattern of staining was generalized in all cases (Fig. 2N, Table 2).

In the body of the stomach, sLe^x expression was noted in four cases. The level of immunostaining varied from weak to strong. The staining included superficial foveolar epithelium (Fig. 2P). The staining distribution was multifocal in all cases studied. The subcellular staining was mainly localized in the cytoplasm of the cells. In the antrum of the stomach, a sparse and moderate expression of sLe^x was observed in only one case. The staining was limited to the cytoplasm of the foveloar superficial epithelium and cells of the pyloric glands (Fig. 2Q, Table 2).

Expression of Terminal α-GalNAc in Dog Gastric Mucosa

In the body of the stomach, expression of terminal α-GalNAc was found in all canine cases, as detected by DBA lectin labeling. The staining was moderate in three cases and intense in the remaining five cases. The staining included superficial foveolar epithelium and parietal cells (Fig. 2S). The staining pattern was generalized in all cases. The subcellular staining was localized in the cytoplasm of the cells. In the antrum of the stomach, generalized expression of terminal α-GalNAc was

Table 2 Terminal α-GalNAc (DBA), type 1 and type 2 Lewis antigens expression in canine gastric mucosa

			Type 1			Type 2		
		DBA	Le^a	Le^b	sLe^a	Le^x	Le^y	sLe^x
Body (n = 8)	Positive immunostaining	8/8	1/8	0/8	0/8	5/8	8/8	4/8
	Immunostaining distribution							
	Sparse	0	1^+	0	0	0	0	0
	Focal	0	0	0	0	0	0	0
	Multifocal	0	0	0	0	$5^{++/+++}$	$4^{+/+++}$	$4^{+/+++}$
	Generalized	$8^{++/+++}$	0	0	0	0	4^{+++}	0
Antrum (n = 6)	Positive immunostaining	6/6	0/6	0/6	0/6	6/6	6/6	1/6
	Immunostaining distribution							
	Sparse	0	0	0	0	0	0	1^{++}
	Focal	0	0	0	0	0	0	0
	Multifocal	0	0	0	0	$4^{++/+++}$	0	0
	Generalized	$6^{++/+++}$	0	0	0	$2^{+/+++}$	$6^{++/+++}$	0

DBA, *Dolichus biflorus* agglutinin.
Intensity variation observed: +weak; ++moderate; +++intense.

detected in all cases. The level of staining was moderate in four cases (Fig. 2T) and intense in the other two. The staining included the foveolar epithelium, gastric pits and pyloric glands. Generally, the superficial pyloric glands showed an intense staining higher than that recorded in the deeper glands; only in one case the staining was identical in both areas. The subcellular staining was localized in the cytoplasm of the cells. (Table 2).

In vitro Binding Assay of *Helicobacter pylori* and NHPH to Canine Gastric Mucosa

The FITC labeled Helicobacter strains were verified for size and morphology in accordance with literature: *H. pylori* strains measured about 2.5–5 μm in length and 0.5–1 μm in width (Fig. 3A,B); The NHPH strains were larger and apparently more tightly coiled spiral-shaped bacteria: ASB1.4 was, approximately 3–6.5 μm long and 0.6–0.7 μm wide (Fig. 3C); CS1 measured about 5–7.5 μm in length and 0.4 in width (Fig. 3D) while R1051 ranged from 5 to 10 μm in length and 0.3 in width (Fig. 3E). R1053 was less spiral, measuring 5–7 μm in length and 0.8–1.2 μm wide (Fig. 3F) (as reviewed by [34]). The length was variable depending on the state of contraction.

Regarding tissue sections from the body of canine stomach, we observed that the *H. pylori* strain 17875*babA1A2* (SabA+) had the highest adhesion. In contrast, the *H. pylori* strain 17875/Leb (BabA+) showed low levels of adhesion. Among the NHPH strains evaluated, ASB1.4 presented the highest adhesion value, followed by CS1 and R1051. The R1053 was the strain which adhered less being possible to observe gastric regions completely devoid of bacterial adhesion (Fig. 4).

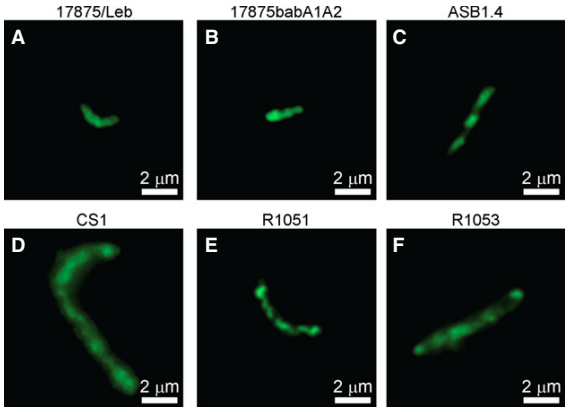


Figure 3 The panel shows the various morphologies of fluorescein-labeled *Helicobacter* spp. strains: (*A*) *H. pylori* 17875/Leb (*B*) *H. pylori* 17875bab*A1A2* (*C*) *H. heilmannii* s.s. ASB1.4 (*D*) *H. felis* CS1 (*E*) *H. bizzozeronii* R1051 (*F*) *H. salomonis* R1053. Magnification 1000×.

In the antral sections, the *H. pylori* strains 17875*babA1A2* and 17875/Leb showed a similar binding behavior, exhibiting very closely means values of adhesion. The quantification revealed that ASB1.4 was the strain that presented highest adhesion values to the canine antrum tissue sections. Additionally, CS1 and R1051 strains adhered similarly to canine antral mucosa, whereas R1053 adhered less (Fig. 5).

The ASB1.4 adhesion value quantified in the antral mucosa was significantly higher from that obtained in the body region of the canine stomach ($p < .05$). No significant differences were found in CS1, R1051, and

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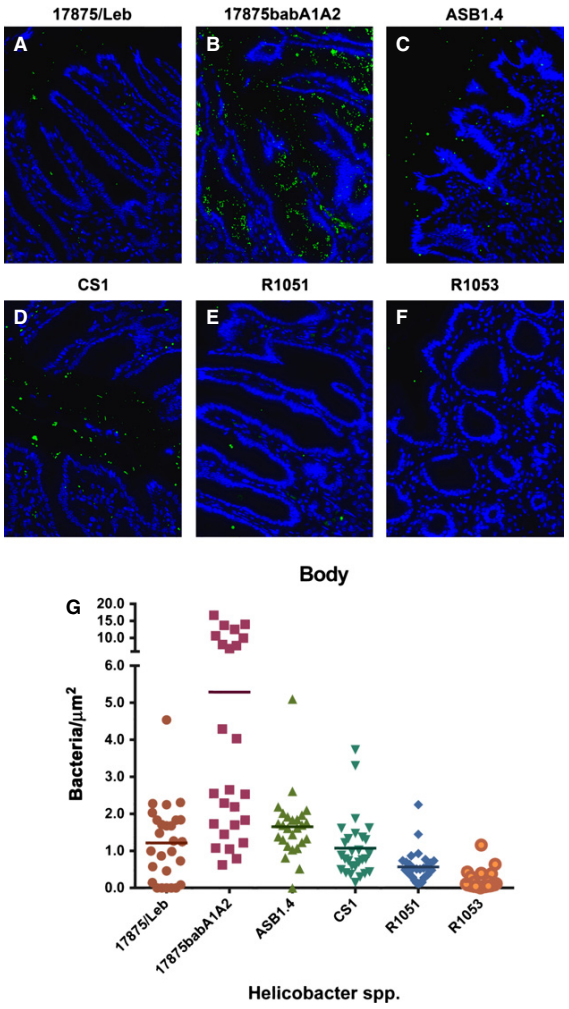


Figure 4 Evaluation of Helicobacter adherence to the body region of the canine gastric mucosa. Adhesion of fluorescein-labeled *H. pylori* model strains: (*A*) 17875/Leb and (*B*) 17875*babA1A2;* Adhesion of fluorescein-labeled Non-*H. pylori* Helicobacters (NHPH) strains: (*C*) ASB1.4, (*D*) CS1, (*E*) R1051and (*F*) R1053. Magnification 200×. (*G*) Quantification of adhesion of the different *Helicobacter* spp. strains. Each symbol represents the mean value obtained by evaluating at least five different fields within the foveolar or the glandular regions, as defined in Materials and Methods. The differences of the mean values between the groups were statistically significant using one-way ANOVA ($p < .0001$).

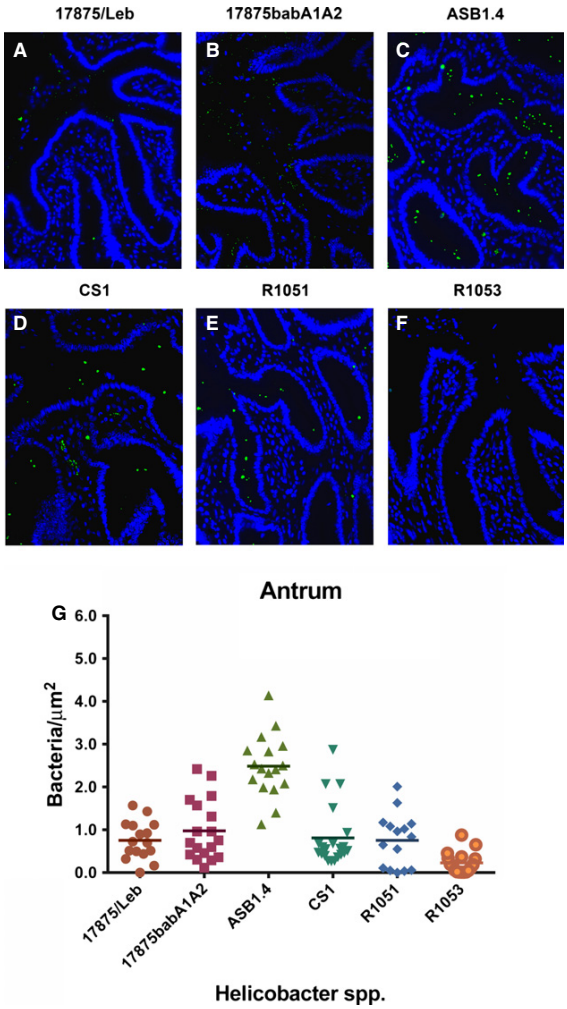


Figure 5 Evaluation of Helicobacter adherence to the antral region of the canine gastric mucosa. Adhesion of fluorescein-labeled *H. pylori* model strains: (*A*) 17875/Leb and (*B*) 17875*babA1A2*; Adhesion of fluorescein-labeled Non-*H. pylori* Helicobacters (NHPHs) strains: (*C*) ASB1.4, (*D*) CS1, (*E*) R1051 and (*F*) R1053. Magnification 200×. (*G*) Quantification of adhesion of the different *Helicobacter* spp. strains. Each symbol represents the mean value obtained by evaluating at least five different fields within the foveolar or glandular regions, as defined in Materials and Methods. The differences of the mean values between the groups were statistically significant using one-way ANOVA ($p < .0001$).

R1053 bacterial adhesion, concerning both canine stomach compartments.

Discussion

In the present study, we have characterized the expression of histo-blood group antigens in the normal canine gastric mucosa to define the gastric glycosylation profile of dogs (Table 2). The expression pattern of type 1 and type 2 Lewis antigens in the canine gastric mucosa revealed a different distribution when compared with the human gastric mucosa. Normal canine mucosa showed minor expression of type 1 Lewis antigens, with absence of Le^b and sLe^a expression and only a sparse expression of Le^a in the body of one of the animals tested. In the human normal gastric mucosa, and

depending on the secretor status of the individual, Le^a and Le^b are highly expressed in the superficial foveolar epithelium [23,35], where MUC5AC mucin is co-expressed [36]. The normal canine mucosa demonstrated expression of Lewis type 2 antigens characterized by expression of Le^x and Le^y, along with a minor expression of sLe^x. In the canine gastric mucosa this expression was mostly observed in the superficial and foveolar epithelium, with Le^y expression extending to the deeper portions of the gastric gland. In the human gastric mucosa, the expression of Lewis type 2 antigens is generally restricted to the deeper part of the gastric glands, where MUC6 mucin is co-expressed [37,38]. Knowing that type 1 fucosylated structures are receptors for *H. pylori* in human stomach, the different expression patterns of type 1 and 2 Lewis antigens observed may play a role in differences in prevalence of gastric helicobacters in both mammalian species.

The pattern of Lewis antigen expression observed in canine gastric mucosa is in agreement with a previous study which reported a prominent representation of type 2 chains in dog intestinal secretions [39].

According to our results, the A-antigen is also extensively expressed in the normal canine gastric mucosa, representing an important component of the dog gastric mucosa glycophenotype (Table 2). This evidence is in accordance with earlier observations documenting that this antigen is expressed in canine gastrointestinal tissues [30]. Interestingly, the pattern of expression of the A-antigen in canine gastric mucosa resembles the reactivity of DBA lectin previously reported in human gastric tissue [40]. However, its putative involvement in Helicobacter adhesion has never been addressed.

Lewis antigens biosynthesis is dependent on the cells repertoire of glycosyltransferases, namely fucosyltransferases. The absence of type 1 Lewis antigens in dog gastric mucosa may be explained by the lack of expression of enzymes with α1,4 fucosyltranferase activity. The human enzymes with this activity, FUT3 and FUT5, seem to have originated late in hominid evolution from duplication of an ancestor Lewis gene present in lower mammals [41]. Moreover, it has been demonstrated that the acceptor substrate specificity of vertebrate α1,3/1,4-fucosyltransferases is determined by a single amino acid in the hypervariable stem domain [42]. Most vertebrate α1,3 fucosyltransferases present a conserved arginine in this domain while a tryptophan residue is present at the same position on human FUT3 and FUT5. Noteworthy, the dog gene orthologous of the human FUT3 and FUT5 gene presents at this position an Arg residue, compatible with α1,3 fucosyltranferase activity and therefore type 2 Lewis synthesis (Fig. S1).

The host glycan receptors are recognized by Helicobacter outer membrane proteins (OMPs) which mediate several important host-pathogen interactions [18]. Recently, it has been shown that NHPH OMPs genes share sequence homology with *H. pylori*. Interestingly, it was found that *H. acinonychis*, a NHPH colonizing big cats, express BabA adhesin [43].

However, among the NHPH strains included in this study, none presented in their genomes orthologues of *H. pylori* adhesins. *H. bizzozeronii* and *H. mustelae* lack homologs of major *H. pylori* virulence genes BabA/B and SabA while *H. suis* lacks BabA/B and SabA/B [44–46]. The genome of *H. heilmannii* s.s. showed that this strain encodes several OMPs but lacks the Bab and Sab adhesins [47].

The absence of homologues of the BabA in these NHPH may underlie the low rate of human infection by these organisms and suggests that other adhesins with different receptor specificity may play a role in the colonization of NHPH. Additionally, the lack of Lewis type 1 antigens expression in the canine gastric mucosa might explain why *H. pylori* rarely colonizes the stomach of dogs.

To determine if the glycosylation profile of canine gastric mucosa was related to the host-adaptation of the different NHPH, we evaluated the binding capacity of some *Helicobacter* species to canine gastric mucosa.

Our results show that *H. heilmannii* s.s. adhered most to the canine gastric mucosa. This result highlights the contribution of adhesion in the canine gastric colonization by this species.

In 2012, *H. heilmannii* was isolated and described as a novel species [34]. The name *H. heilmannii* sensu stricto (s.s.) was proposed to refer to the novel *Helicobacter* species and the term *H. heilmannii* sensu lato (s.l.) to refer to the whole group NHPH [12]. Since the discovery and description of *H. heilmannii* s.s. is still relatively recent, the majority of the epidemiological data available does not comprise the identification of this particular species in the stomach of dogs and cats. This impairs the comparison of the adhesion capacity achieved in this experiment with the spontaneous incidence of *H. heilmannii* s.s. in dogs.

We also found that the *H. heilmannii* s.s. adhesion levels were higher in the antrum than in the body region of canine stomach ($p < .05$). In agreement with our observations, Joosten et al. [48] recently studied bacterium-host interactions of 9 *H. heilmannii* s.s. strains in Mongolian gerbils and concluded that, in general, the colonization capacity in the fundus was lower than in the antrum for all strains tested. Additionally, a clear association was observed between the colonization capacity of the *H. heilmannii* s.s. strains and the gastric

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inflammation scores in the antrum of the stomach. In dogs, while many studies reported that the fundus and body present higher bacteria density and higher probability to find *Helicobacter* spp. [8,49,50] others found no significant differences between the density of NHPH in the fundus, body and antrum [31,51–54]. The discrepancies in the results regarding the spatial distribution of *Helicobacter* spp. in canine gastric mucosa can be attributed to the different laboratorial approaches and diagnostic methodologies used by the various research groups.

The other NHPH strains tested showed lower adhesion levels to the canine gastric mucosa. In comparison to *H. heilmannii* s.s., *H. felis* has a lower adhesion capacity, followed by *H. bizzozeronii* and *H. salomonis* and this trend was observed in the body as well as in the antrum.

The two *H. pylori* strains used in this study are model strains frequently used in adhesion studies due to their well-defined binding properties. In both stomach regions, the *H. pylori* strain 17875/Leb, which is a BabA positive strain, adhered less than *H. heilmannii* s.s. This can be explained by the fact that dogs do not express Lewis type 1 antigens, including the BabA-ligand Le^b. The *H. pylori* 17875*babA1A2*, which is a SabA positive strain, adhered considerably in the body region. This result is in accordance with the concomitant observed expression of sLe^x, the ligand of the SabA adhesin. However, in vivo and probably due to competitive factors between bacteria and/or different immunological responses of the host, *H. pylori* often fails to colonize the canine gastric mucosa.

Our results support that the differences in gastric mucosa glycosylation profile play a role in the host adaptation of gastric *Helicobacter* species. The defined canine gastric mucosa glycophenotype indicates that adhesion of NHPH involves different adhesion mechanisms mediated by proteins with alternative receptor specificity. The knowledge of the adhesion abilities of different Helicobacter strains to canine gastric mucosa is of outmost relevance for the understanding of the physiopathology of Helicobacter infection in the dogs.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Alignment of the peptide sequences of the predicted galactoside 3(4)-L-fucosyltransferase-like from *Canis lupus familiaris* and the *Homo sapiens* α1,3 and α1,3/1,4-fucosyltransferase FUT3.

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gi|345786697| Predicted: galactoside 3(4)-L-fucosyltransferase-like [Canis lupus familiaris]  MDPHILAKIRCPWRHYLFGVLFQLLLALCFFSYMRWSQEEPVWFSMSRTNTAESPATAPN 60
gi|121137|FUT3: galactoside 3(4)-L-fucosyltransferase-like [Homo sapiens]                    MDPLGAAKPQWPWRRCLAALLFQLLVAVCFFSYLRVSRDDAT----------GS-PRAPS 49
                                                                                             ***   ** : ***: * .:*****:*:*****:* *::: .          *   **.

gi|345786697| Predicted: galactoside 3(4)-L-fucosyltransferase-like [Canis lupus familiaris]  GSAGPVTSQGAPCQATRGSSACRPLLLLLWTWPFHHPVALSPCSDMWPGTTDCRMTANRS 120
gi|121137|FUT3: galactoside 3(4)-L-fucosyltransferase-like [Homo sapiens]                    GSSRQ-----------DTTPTRPTLLILLWTWPFHIPVALSRCSEMVPGTADCHITADRK 98
                                                                                             **:               : :   **:******** ***** **:* ***:**::**:*.

gi|345786697| Predicted: galactoside 3(4)-L-fucosyltransferase-like [Canis lupus familiaris]  VYPEADAVIVHHEFYSYRPKEQLPTSPRPPGQRWVWFSMESPSHCAQLKALDGYFNLTMS 180
gi|121137|FUT3: galactoside 3(4)-L-fucosyltransferase-like [Homo sapiens]                    VYPQADTVIVHHWDMSNPKSRLPPSPRPQGQRWIWFNLEPPPNCQHLEALDRYFNLTMS 158
                                                                                             ***:**:*:*** ::  .**.:** **** ****:**.:* * .* :*:*** *******

gi|345786697| Predicted: galactoside 3(4)-L-fucosyltransferase-like [Canis lupus familiaris]  YRRDSDIFTPYGWLEPWPGQPAAPLVNLSAKTELVAWVVSNWREDSVRVRYYHKLQAHVQ 240
gi|121137|FUT3: galactoside 3(4)-L-fucosyltransferase-like [Homo sapiens]                    YRSDSDIFTPYGWLEPWSGQPAHPPLNLSAKTELVAWAVSNWKPDSARVRYYQSLQAHLK 218
                                                                                             ** ************** **** * :***********.****: **.*****:.****::

gi|345786697| Predicted: galactoside 3(4)-L-fucosyltransferase-like [Canis lupus familiaris]  VDVYGQRHKPLPQRNMIQTLSQYKFYLAFENSLHRDYITEKLWRNALDAWAVPVVLGPSR 300
gi|121137|FUT3: galactoside 3(4)-L-fucosyltransferase-like [Homo sapiens]                    VDVYGRSHKPLPKGTMMETLSRYKFYLAFENSLHPDYITEKLWRNALEAWAVPVVLGPSR 278
                                                                                             *****: *****: .*::***:************ ************:************

gi|345786697| Predicted: galactoside 3(4)-L-fucosyltransferase-like [Canis lupus familiaris]  KSYEQFLPADAFIHVDDFQNPKDLAQYLLSLDKDDARYLAYFRWRETLRPRTFSWSLMFC 360
gi|121137|FUT3: galactoside 3(4)-L-fucosyltransferase-like [Homo sapiens]                    SNYERFLPPDAFIHVDDFQSPKDLARYLQELDKDHARYLSYFRWRETLRPRSFSWALDFC 338
                                                                                             ..**:*** **********.*****:** .****.****:***********:***:* **

gi|345786697| Predicted: galactoside 3(4)-L-fucosyltransferase-like [Canis lupus familiaris]  KACWRLQQESAYQTVPSIASWFT 383
gi|121137|FUT3: galactoside 3(4)-L-fucosyltransferase-like [Homo sapiens]                    KACWKLQQESRYQTVRSIAAWFT 361
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- Epidemiological characterization of *Helicobacter spp.* in canine gastric mucosa

CHAPTER 3

Paper III

Epidemiological characterization of the Helicobacter spp. present in canine gastric mucosa

Irina Amorim, Annemieke Smet, Odete Alves, Silvia Teixeira, Ana Laura Saraiva, Freddy Haesebrouck, Celso Reis, Fátima Gärtner

(In preparation)

Epidemiological characterization of the *Helicobacter spp.* present in canine gastric mucosa

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Key-words: Canine gastric mucosa; dogs; non-*Helicobacter pylori* helicobacters (NHPH); histochemistry; immunohistochemistry (IHC); polymerase chain reaction (PCR); stomach.

Abstract

To determine the prevalence of *Helicobacter* species and the gastric histopathological findings, gastric mucosa samples of 69 dogs were evaluated. *Helicobacter* species were detected in 59 dogs (85.5%) by at least one detection method. Histological, histochemical and immunohistochemical evaluations revealed *Helicobacter spp*. in 45 (65.2%), 52 (75.4%) and in 57 (82.6%) dogs, respectively. Bacteria were detected by PCR analysis in 33 (47.8%) dogs. *H. heilmannii-like* organisms were the most commonly found being identified in 22 animals (66.7%) and predominantly in the antral gastric region. *H. salomonis* was the second more prevalent species (51.5%) although it was mainly found in association with other than NHPH and in the body gastric region. *H. bizzozeronni and H. felis* were the less frequent species and *H. cynogastricus* and *H. baculiformis* were not detected at all. It was concluded that, despite the high incidence and worldwide distribution of NHPH, geographic variations partially account for the prevalence of a specific *Helicobacter* species in the dog stomach.

Introduction

The genus *Helicobacter* is composed of at least 32 species (Haesebrouck *et al.*, 2009). Among these, *Helicobacter pylori* (*H. pylori*) is consider an important pathogen whose natural host is man but its presence in the canine stomach has been rarely reported (Buczolits *et al.*, 2003; Chung *et al.*, 2014; Ekman *et al.*, 2013).

A large number of non-*Helicobacter pylori Helicobacter* (NHPH) species have been also recognized in humans and in several animals. Previously, NHPHs were generally referred to as "*H. heilmannii*" which was further subdivided in two taxa, types 1 and 2 (Haesebrouck *et al.*, 2009). Microorganisms referred to as *H. heilmannii* type 1 are identical to *H. suis*, a species colonizing the stomachs of pigs. The former *H. heilmannii* type 2 represents a group of species known to colonize the gastric mucosa of dogs: *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. cynogastricus*, *H. baculiformis* (Haesebrouck *et al.* 2009) and a bacterium which was, in 2004, given the provisional name "*Candidatus H. heilmannii*" because, at that time, it could not be cultured *in vitro* (O'Rourke *et al.*, 2004). In 2011, a description of *H. heilmannii* as a novel species was defined (Smet *et al.*, 2012). The name *H. heilmannii sensu stricto* (s.s.) was proposed to refer to the novel *Helicobacter* species and the term *H. heilmannii sensu lato* (s.l.) to refer to the whole NHPH group (Haesebrouck *et al.*, 2011).

NHPHs are present in 67–86% of clinically healthy dogs, in 61–100% of animals presenting chronic vomiting (Geyer *et al.*, 1993; Hermanns *et al.*, 1995; Hwang *et al.*, 2002; Lecoindre P., 1995; Polanco *et al.*, 2011) and in about 100% of laboratory Beagle dogs and dogs from local shelters (Eaton *et al.*, 1996; Henry *et al.*, 1987; Simpson *et al.*, 1999). The predominant gastric *Helicobacter spp.* in dogs are *H. felis*, *H. bizzozeronii* and *H. heilmannii sensu stricto* (*s.s.*), while *H. salomonis* is less often detected and the prevalence of *H. cynogastricus* and *H. baculiformis* has not yet been studied (Baele *et al.*, 2009; Haesebrouck *et al.*, 2009; van den Bulck *et al.*, 2005; Van den Bulck *et al.*, 2006).

The diagnostic methods used for *Helicobacter spp.* can be non-invasive and invasive (Prachasilpchai *et al.*, 2007). Non-invasive methods like serology or detection of bacterial DNA and antigens in stools do not require a gastric biopsy or anaesthesia. The invasive methods, like bacterial cultures, histopathology, smears, electron microscopy or polymerase chain reaction (PCR), require a gastric biopsy, which is frequently obtained through endoscopy under anaesthesia or during necropsies. Typically *Helicobacter spp*. organisms are not easily visualized with the haematoxylin and eosin (HE) stain and so, their direct observation in biopsied specimens is highlighted by the use of special stains, such as the modified Giemsa (MG) stain. More elaborate and sensitive detection methods such as immunohistochemistry (IHC) or polymerase chain reaction (PCR) are research tools rarely used in a diagnostic setting.

Several investigations have discussed the prevalence of *Helicobacter* spp. in dogs (Buczolits *et al.*, 2003; Geyer *et al.*, 1993; Henry *et al.*, 1987; Hermanns *et al.*, 1995; Polanco *et al.*, 2011) but few determine the specific species present in the canine stomach (Chung *et al.*, 2014; Jalava *et al.*, 1998; Priestnall *et al.*, 2004; van den Bulck *et al.*, 2005). The accurate identification of the *Helicobacter spp.* is essential in order to determine the prevalence and clinical significance of all taxa.

The aim of this study was to determine the prevalence and to identify the *Helicobacter* species present in distinct stomach regions of the canine stomach (body and antrum) using histological, histochemistry, immunohistochemistry and molecular diagnostic techniques. The degree of colonization was characterized and correlated with the respective histopathological changes in canine gastric mucosa.

Materials and Methods

Sample collection

The gastric tissues were obtained from 69 dogs (45 male and 24 female, ranging in age from 3 months to 15 years). The samples were randomly selected from the archives of the Laboratory of Veterinary Pathology, ICBAS-UP (Portugal) where they were received between 2010 and 2013. Twenty samples were collected during endoscopic procedures, five during surgery and 44 during necropsy examinations. When available, the detailed description of each case was recorded and included information such as breed, sex, age and clinical signs. Only the gastric samples in good condition of preservation were included in this study.

Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Three consecutive sections 3 µm thick were made, one being stained with HE, other with a MG stain and the third was used for the immunohistochemical study.

Sample evaluation

Histopathological parameters such as alterations in cellularity, fibrosis of the *lamina propria* and gland atrophy were analysed according to the World Small Animal Veterinary Association (WSAVA) guidelines (Day *et al.*, 2008). The degree of morphological features and inflammatory changes was graded as normal, mild, moderate or marked by using the available WSAVA gastrointestinal standardization visual analogue (Day *et al.*, 2008).

The microscopic evaluation was performed by analysing the entire section of the gastric tissue. The presence of *Helicobacter spp.* organisms was assessed by HE and MG stains and by IHQ. A dog was classified as *Helicobacter* spp. positive whenever one of these methods gave a positive result. Additionally, bacterial density colonization was quantified: +, few organisms (<10 organisms/400x); ++, moderate number of organisms (10 to 50 organisms/400x); +++, large number of organisms (>50 organisms/400x) (Prachasilpchai *et al.*, 2007).

All the cases were independently examined by two pathologists and when there was a divergence of opinion, an agreed diagnosis was reached through simultaneous observation in a multihead microscope.

Immunohistochemistry

For the immunohistochemical study, sections were deparaffinised, hydrated and antigen retrieval was performed in a pressure cooker in 10 mmol/L sodium citrate buffer, pH 6.0, for 2 minutes (min). Slides were cooled for 10 min at room temperature and rinsed twice in triphosphate buffered saline (TBS) for 5 min. The NovolinkTM Max-Polymer detection system (Novocastra, Newcastle, UK) was used for visualisation, according to the manufacturer´s instructions. After blocking endogenous peroxidase with 3% hydrogen peroxide in methanol for 10 min, sections were incubated overnight at 4ºC, with a polyclonal antiserum specific for *H. pylori* (RBK012; Zytomed, German) and shows immunoreactivity with a wide range of bacteria belonging to the *Helicobacter* genus. Sections were rinsed with TBS between each step of the procedure. Colour was developed for up to 7 min at room temperature with 3,3'-diamino-benzidine (DAB) (Sigma, St. Louis, MO) and sections were then lightly counterstained with haematoxylin, dehydrated and mounted. Positive immunoreactivity was recorded as a distinct golden-brown labelling of the bacteria located on mucosal surface, in gastric pits or glands and in parietal cells.

Extraction, PCR amplification and sequencing of DNA

DNA was extracted from gastric biopsies embedded in paraffin (5 slices of 20 μM) using a DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's instructions. Helicobacter-species specific qPCRs based on a short fragment of the urease A and B genes were developed for the identification of *H. heilmannii*, *H. felis*, *H. bizzozeronii* and *H. salomonis.* For generation of standards for each qPCR, a large part of the ureAB gene cluster from *H. heilmannii* ASB1 (1224bp), *H. felis* CS1 (1228bp), *H. salomonis* R1053 (1224bp) and *H. bizzozeronii* R1051 (1230bp) was amplified using primers U430F and

U1735R, as described previously (O'Rourke et al., 2004). The standard consisted of 10-fold-dilutions starting at 108 PCR amplicons for each 10 µl of reaction mixture. One µl of extracted DNA template was suspended in a 10 µl reaction mixture consisting of 0.25 µl of forward and reverse primers (Table 1) located within each standard, 3.5 µl HPLC water and 5 µl SensiMix™ SYBR No-ROX (Bioline Reagents Ltd, UK). Both standards and samples were run in duplicate on a CFX96™ RT-PCR System with a C1000 Thermal Cycler (Bio-Rad, Hercules CA, USA). The Bio-Rad CFX Manager (version 1.6) software was used for calculation of threshold cycles (Ct)-values and melting curve analysis of amplified DNA. The average values of the duplicates were used for quantification of *Helicobacter* DNA in the tissue samples.

Statistical Analysis

Pearson's chi-square test and Fisher's exact test were used to determine the dependence between two categories. p values < 0.05 were considered as statistically significant. Statistical analysis was performed using the statistical package SPSS 16.0 (SPSS Inc., Produtos e Serviços de Estatística Lda, Lisbon, Portugal).

Results

A total of 117 gastric samples (66 from the body region and 51 from the antrum region) were analysed.

Among the 69 animals, both gastric regions were available for evaluation in 48 dogs whereas only body or antrum regions were available in 18 and three dogs, respectively.

The number of body gastric samples classified as inadequate, too superficial and adequate were one, eight, and 57, respectively. Regarding the antral samples, three were considered too superficial and 48 adequate. All the samples classified as inadequate or too superficial, regardless of the gastric location, were collected by endoscopy.

Of the 69 dogs, only one presented a normal histopathological mucosa with absence of spiral-shaped organisms. Regardless of the stomach location, both normal gastric mucosa and presence of bacteria, were observed in two dogs (2.9%). The remaining animals, presented histopathological changes representative of gastritis (66/69 or 95.7%) (Table 2). Additionally, a single type of gastritis (lymphoplasmacytic) was found in 18 dogs while in the others, a combination of different types was present. On the basis of histopathological changes in gastric mucosa, we diagnosed a mild to moderate chronic gastritis in 88.4%

(61/69) affecting the gastric body of 51.5% (34/66) and the antral region of 92.2% (47/51) of the animals.

Both mild to moderate epithelial injuries and mild to moderate intraepithelial lymphocytes were found in 88.4% (61/69).

Gastric mucosal atrophy, glandular nesting or fibrosis was present in 44.9% dogs (31/69). In 18.2% of the animals this alteration was observed in the body region (12/66) and in 47.1% in the antrum region (24/51) of the canine stomach.

Abnormal neutrophilic infiltration was only detected in the stomach antrum of two animals: in one, this alteration was mild and in the other, it was marked and associated with gastric ulceration. Other inflammatory cells, consisting in mild infiltration of mast cells, were observed in the body region of four animals and in the antrum of two animals.

Among all the animals, 87.0% were positive (60/69) and 13.0% were negative for *Helicobacter spp*. (9/69), irrespective of the test used to detect the bacteria. Regardless of the stomach location, the presence of *Helicobacter spp*. was observed using HE, MG and IMC in 65.2% (45/69), 75.4% (52/69) and 82.6% (57/69) of the dogs, respectively. With the routine staining, bacteria were detected in 62.1% of body samples (41/66) and in 70.6% of antral samples (36/51). Utilizing the MG stain, *Helicobacter spp.* was identified in 68.2% of body samples (45/66) and in 78.4% of the antral samples (40/51). The presence of *Helicobacter* spp. antigen in the body region of the canine stomach was 84.9% (56/66) and in the antrum region it was 80.4% (41/51) (Table 3). Immunohistochemistry was the method that most easily identified the bacteria in the intracellular location.

Helicobacter spp. organisms were detected by PCR in 47.8% of the animals (33/69) (Table 3). Since the majority of the specific *H. heilmannii spp.* PCR-positive samples, the amplicons showed ≥ 92% homology with *H. heilmanni s.s.*, these cases were reclassified as *H. heilmannii-like.*

In 51.5% (17/33) of the canine positive samples, only one *Helicobacter* species was identified while mixed infections were detected in 48.5% (16/33) (Table 2). *H. heilmannii-like* organisms were the most commonly found (22/33 or 66.7%), being identified in ten dogs as a single infection and in 12 dogs as mixed infections. *H. salomonis* was the second most prevalent species (17/33 or 51.5%) although it was mainly found in association with other NHPHs (42%) rather than alone (9.1%). Equal proportions of *H. felis* and *H. bizzozeronnii* were detected (6/33 or 18.2%), either as single (6.3%) or mixed infections (12.1%), respectively. Mixed infections by *H. heilmanni* plus *H. salomonis* were the most encountered (33.3%) (Table 2). In the body area, the species most frequently identified was *H. salomonis* (44.0%) whereas in the antrum the most prevalent species was *H. heilmannii-like* (57.7%) (Table 4).

No positive PCR-samples for *H. cynogastricus* and *H. baculiformis* were obtained in this study.

There was a significant correlation between the presence of the bacteria and both mild to moderate intraepithelial lymphocyte infiltration and mild to moderate epithelial injury of the canine stomach ($p<0.05$). No statistically significantly correlation between *Helicobacter spp.* infection and gastric mucosal atrophy or fibrosis, *lamina propria* lymphoplasmacytic infiltration or lymphofollicular hyperplasia were found. Nor was a correlation observed between mucosal atrophy or fibrosis and the age of the dogs included in the study and no significant differences were detected regarding the bacterial colonisation density between both stomach regions ($p > 0.05$).

The number of *Helicobacter*-positive cases detected with the different methods differ significantly ($p<0.05$). Positive IHC results did not differ significantly across each stomach region ($p > 0.05$), while the numbers obtained with HE, GM and PCR differ significantly between the body and the antrum ($p < 0.05$).

Discussion

In this study, a high prevalence of gastritis was observed (95.7%). These results are in agreement with others reporting the occurrence of gastritis as a common finding in dogs (Happonen *et al.*, 1996; Neiger and Simpson, 2000; Polanco *et al.*, 2011) . In contrast, gastric erosions or ulcers were seldom found in these animals. In agreement with the literature, a relationship between canine gastritis and *Helicobacter spp.* infection has not been established (Eaton *et al.*, 1996; Hermanns *et al.*, 1995; Polanco *et al.*, 2011; Shabestari *et al.*, 2008). However, canine NHPH infection was significantly accompanied by mild to moderate intraepithelial lymphocyte infiltration and mild to moderate gastric epithelial injury, regardless of the stomach location.

In the present study, NHPH infection was determined by at least four methods (HE; MGS, IHC and PCR) and a prevalence of 87.0% in dogs was detected. These results are in accordance with the available literature, which documented high prevalence of NHPH in canine gastric mucosa (Hwang *et al.*, 2002; Moutinho *et al.*, 2007; Shabestari *et al.*, 2008; van den Bulck *et al.*, 2005; Wiinberg *et al.*, 2005). Since most of these dogs were accompanied by none or little clinical information, it is impossible to compare these results with others in which this high prevalence was observed both in healthy dogs (Moutinho *et al.*, 2007) and those presenting gastrointestinal signs (Happonen *et al.*, 1996; Hwang *et al.*, 2002).

In a previous investigation, the amount of *Helicobacter*-positive cases detected with HE staining was 17.5% (Prachasilpchai *et al.*, 2007). In the current study, all the samples were examined by two pathologists used to identify *Helicobacter* spp. organisms under the routine staining. Therefore, it is reasonable to assume that the amount of HE-positive cases obtained in our study (65.2%) is probably higher than that generally expected.

In agreement with previous studies, NHPH were often observed not only in the superficial mucus and within the gastric glands but also in the intracellular location, more specifically in the cytoplasm of parietal cells (Happonen *et al.*, 1996; Scanziani *et al.*, 2001; Wiinberg *et al.*, 2005). *Helicobacter* organisms present in this particular subcellular location are difficult to identify with HE and MG stains due to the cytoplasm granulation of the parietal cells. Therefore, the IHC remains a very valuable technique in the identification of NHPH within the parietal cells of the gastric body. Additionally, this was the method that demonstrated higher *Helicobacter*-positive values (82.6%). In agreement, other authors also showed that commercially available antibodies against *H. pylori* are very sensitive in the detection of *Helicobacters* in paraffin embedded samples from dogs' stomachs (Gombač *et al.*, 2010; Prachasilpchai *et al.*, 2007; Scanziani *et al.*, 2001). Prachasilpchai *et al.* (2007) also highlighted that IHC constitutes the best diagnostic tool for formalin-fixed samples (Prachasilpchai *et al.*, 2007).

Previous studies reported no statistically significant difference between the detection of *Helicobacter* organisms by IHC and PCR techniques ($p > 0.05$) (Prachasilpchai *et al.*, 2007). According to Chung *et al.* (2014), the PCR assay has higher sensitivity and specificity than the other methods (Chung *et al.*, 2014). However, in this study, the prevalence rate of *Helicobacter spp.* obtained with the PCR was the lowest (47.8%). Previous investigations have shown that formalin fixation and paraffin embedding (FFPE) hamper PCR analysis (Farrugia *et al.*, 2010; Gilbert *et al.*, 2007). Later, Sjödin *et al.* (2011) compare the efficiency of the DNA amplification from fresh (n=28) and FFPE (n=28) samples in the identification of *Helicobacter spp.* from different organs (feline stomach, duodenum, liver and pancreas) and concluded that the mean value of DNA concentration achieved was higher when obtained from fresh tissues. Additionally, *Helicobacter* DNA from FFPE was only detected in the stomach samples (6/28) while *Helicobacter* DNA from fresh tissues was detected in a higher number of samples as well as in other organs (18/28) (Sjodin *et al.*, 2011).

DNA analysis of FFPE tissue samples using PCR may be compromised due to DNA fragmentation, inhibiting substances, or a combination of both. The formalin fixation may cause DNA fragmentation as well as partial destruction of DNA (Gilbert *et al.*, 2007; Miething *et al.*, 2006) and the PCR reactions may also be inhibited by formalin residue (Farrugia *et al.*, 2010). The negative effects of formalin are directly related to the duration of the fixation (Miething *et al.*, 2006). Although the samples included in this study were all

processed in the same laboratory and therefore subjected to the same standard protocols, there are other factors beyond our control that might influence the technique, such as the fixation time since the sample collection until its arrival at the laboratory.

Different molecular approaches for the identification of NHPH species have been addressed (Chung *et al.*, 2014; Polanco *et al.*, 2011; Prachasilpchai *et al.*, 2007; Priestnall *et al.*, 2004). However, due to the high homology verified between NHPH species, species differentiation based on some particular methods may not be so reliable. As previously reported, mixed infections are a common and interesting finding in NHPH infection. In contrast to *H. pylori* in human gastric mucosa, mixed colonisation of the same niche by two or more species often occurs and may provide conditions for recombination and genetic exchange between species and this can explain the variable degree of homology detected among the *H. heilmannii s.s*. organisms.

According to our results, in these dogs *H. heilmannii-like* organisms were the most commonly found (22/33 or 66.7%), being identified in 10 dogs as a single infection and in 12 dogs as mixed infections. *H. salomonis* was the second more prevalent species (17/33 or 51.5%) although it was mainly found in association with other than NHPH (42.0%) rather than alone (9.1%). Equal proportions of *H. felis* and *H. bizzozeronnii* were detected (6/33 or 18.2%), either as single (6.3%) or mixed infections (12.1%), respectively.

Some authors describe *H. felis* as the most commonly found in dogs (Eaton *et al.*, 1996). In contrast, in dogs from Finland, Switzerland, the United States and Denmark *H. bizzozeronii* and *H. salomonis* were the most common, followed by *H. felis* and *H. heilmannii* (Jalava *et al.*, 1998; Priestnall *et al.*, 2004; Wiinberg *et al.*, 2005). In dogs from Belgium, Van den Bulck *et al.* (2005) also documented that *H. bizzozeronii* was the most prevalent *Helicobacter* species, as it was identified in 22 dogs (20.0%) as a single infection and in 55 dogs (50.0%) as mixed infections. Single infections with *H. felis* or HLO135 were sporadically encountered, while single infections with *H. salomonis* and "*Candidatus Helicobacter suis*" were not identified in any sample (van den Bulck *et al.*, 2005). To the best of our knowledge, the prevalence of *H. cynogastricus* and *H. baculiformis* in canine stomach has not yet been investigated and neither of these were detected in this study.

The prevalence of NHPH was different in both canine stomach regions. Recently, the *in vitro* binding capacity of some *Helicobacter* species to canine gastric mucosa was assessed and it was concluded that *H. heilmannii* s.s. was the species that adhered the most, followed by *H. felis*, *H. bizzozzeronnii* and *H. salomonis*, and this tendency was observed in both stomach compartments (Amorim *et al.*, 2014). Despite its low binding capacity *in vitro, H. salomonis* appears to be more effective in colonizing the body stomach of the dog. This results support the hypothesis that binding is one aspect, among several

others, in the colonization process. Regarding the antrum region, the *in vitro* binding assays are in accordance with the results herein obtained and support the high prevalence of *H. heilmannii-like* organisms in this specific location (Amorim *et al.*, 2014).

Given these results and the proportion of dogs showing the *H. heilmannii-like* and *H. salomonis* bacterial combination, it seems plausible that direct competition between at least these two species may occur in order to achieve the successful colonization of the gastric niche in the dog. Furthermore, the high percentage of mixed infections comprising *H. salomonis* may suggest that the colonization capacity of this specific species in the gastric context may be enhanced when associated with other NHPH. However, further studies are needed in order to clarify these hypotheses.

Taken together, all these results suggest that, despite the high incidence and worldwide distribution of NHPH, geographic variations amongst other factors, partially account for the prevalence of a specific *Helicobacter* species in the dog stomach.

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Table 1 - List of primers used for qPCR

BFHfel_F2	gCT ggT ggC ATC gAT ACg CAT
BFHfel_R2	TTT TTA gAT TAg CgC gTC Cgg gA
HH_FQ_PAR	ggC TCT gCg TAg gAC CTg CTA CAg AAg CTC TC
HH_RQ_PAR	ggC TgT Agg gAT TTg TTg Agg AgA AAT g
Hsal_FQ_PAR	CTC TTA TgA gTT ggA CTT ggT gCT CAC CAA T
Hsal_RQ_PAR	TTT gCC ATC TTT AAT TCC AAT gTC ggC
Hbizz_PAR_FQ	AAT CTT TgC gTg ggC CCT gCT ACT gAg
Hbizz_PAR_RQ	CTg gCA AAT gCT gTg ggg ATT TgT Tgg

Table 2 - Table summarising the histopathological alterations and colonisation density observed in the canine stomach, according to the respective positive NHPH species-specific PCR results, positive genus results and negative results.

	PCR positive- results with specific species identification, regardless gastric location (percent&number) (n=33)									**Positive results for *Helicobacter spp.*** (percent& number) (n=27)	**Negative results** (percent & number) (n=9)
	Hh (n=10)	*Hf* (n=2)	*Hb* (n=2)	*Hs* (n=3)	*Hf+Hb* (n=1)	*Hh+Hs* (n=11)	*Hh+Hf* (n=1)	*Hb+Hs* (n=1)	*Hf+Hb+Hs* (n=2)		
Histopathology grading (Day *et al.*, 2008)											
Normal	3.0 (1/33)	0	0	3.0 (1/33)	0	0	0	0	0	7.4 (2/27)	11.1 (1/9)
Mild gastritis	15.2 (5/33)	3.0 (1/33)	3.0 (1/33)	6.1 (2/33)	0	18.2 (6/33)	3.0 (1/33)	3.0 (1/33)	6.1 (2/33)	44.4 (12/27)	33.3 (3/9)
Moderate gastritis	12.1 (4/33)	3.0 (1/33)	3.0 (1/33)	0	3.0 (1/33)	9.1 (3/33)	0	0	0	48.1 (13/27)	44.4 (4/9)
Marked gastritis	0	0	0	0	0	6.1 (2/33)	0	0	0	0	11.1 (1/9)
Epithelial injury											
Mild	27.3 (9/33)	3.0 (1/33)	0	9.1 (3/33)	3.0 (1/33)	30.3 (10/33)	0	3.0 1/3(3)	6.1 (2/33)	77.8 (21/27)	33.3 (3/9)
Moderate	3.0 (1/33)	3.0 (1/33)	6.1 (2/33)	0	0	0	3.0 (1/33)	0	0	14.8 (4/27)	11.1 (1/9)
Fibrosis/mucosal atrophy											
Mild	18.2 (6/33)	6.1 (2/33)	6.1 (2/33)	6.1 (2/33)	0	18.2 (6/33)	0	0	3.0 (1/33)	55.6 (15/27)	33.3 (3/9)
Moderate	3.0 (1/33)	0	0	0	3.0 (1/33)	0	0	0	0	3.7 (1/27)	11.1 (1/9)
Intraepithelial lymphocytes											
Mild	15.2 (5/33)	3.0 (1/33)	6.1 (2/33)	6.1 (2/33)	0	15.2 (5/33)	0	0	6.1 (2/33)	29.6 (8/27)	11.1 (1/9)
Moderate	3.0 (1/33)	3.0 (1/33)	0	0	3.0 (1/33)	9.1 (3/33)	0	0	0	14.8 (4/27)	0
Lymphofollicular hyperplasia											
Mild	6.1 (2/33)	0	0	0	3.0 (1/33)	0	0	0	0	29.6 (8/27)	11.1 (1/9)
Bacterial density (based on the IHC results)											NA
+	0	0	0	0	0	0	0	0	0	14.8 (4/27)	
++	3.0 (1/33)	0	0	0	3.0 (1/33)	0	0	0	0	25.9 (7/27)	
+++	24.2 (8/33)	6.1 (2/33)	6.1 (2/33)	9.1 (3/33)	0	33.3 (11/33)	3.0 (1/33)	3.0 (1/33)	6.1 (2/33)	51.9 (14/27)	

Legend: *Hh, H. heilmannii-like; Hf, H.felis; Hb, H. bizozzeronni; Hs, H. salomonis.*
Bacterial density: +, few organisms; ++, moderate number of organisms; +++, large number of organisms. NA: not applicable

Table 3 – Detection of *Helicobacter spp.* in the different stomach compartments of the canine stomach recurring to different diagnostic methods.

Gastric region	**Detection methods** Positive (percent & number)			
	HE	MGS	IHC*	PCR
Body (n=66)	62.1 (41/66)	68.2 (45/66)	84.8 (56/66)	37.9 (25/66)
Antrum (n=51)	70.6 (36/51)	78.4 (40/51)	80.4 (41/51)	51.0 (41/51)

* The positive results obtained with IHC did not differ significantly across each stomach region ($p > 0.05$).

Table 4 - Specific *Helicobacter* species detected by PCR in the different stomach compartments of the canine stomach.

Specific PCR-*Helicobacter spp.* positive results	**Gastric region** (percent & number)	
	Body (n=25)	Antrum (n=26)
H. heilmannii-like	24.0 (6/25)	57.7 (15/26)
H. salomonis	44.0 (11/25)	7.7 (2/26)
H. felis	8.0 (2/25)	3.8 (1/26)
H. bizzozeronnii	4.0 (1/25)	11.5 (3/26)
H. felis + H. bizzozeronnii	4.0 (1/25)	3.8 (1/26)
H. heilmannii-like + H. salomonis	8.0 (2/25)	11.5 (3/26)
H. heilmannii-like + H. felis	4.0 (1/25)	3.8 (1/26)
H. felis + H. salomonis	4.0 (1/25)	0

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- Molecular features of canine gastric lesions

CHAPTER 4

Paper IV

An immunohistochemical study of canine spontaneous gastric polyps

Irina Amorim, Marian A Taulescu, Andreia Ferreira, Alexandra Rêma, Celso A Reis, Augusto M. Faustino, Cornel Cătoi, Fátima Gärtner

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DIAGNOSTIC PATHOLOGY

SHORT REPORT Open Access

An immunohistochemical study of canine spontaneous gastric polyps

Irina Amorim[1,2†], Marian A Taulescu[3†], Andreia Ferreira[1,4], Alexandra Rêma[1], Celso A Reis[1,2,5], Augusto M Faustino[1], Cornel Cătoi[3] and Fátima Gärtner[1,2*]

Abstract

Background: Gastric polyps (GP) are characterised by luminal overgrowths projecting above the plane of the mucosal surface that can be classified as non-neoplastic and neoplastic lesions. In humans, recent studies have drawn attention to the malignant potential of some of these lesions. However, gastric polyps are uncommon lesions in dogs.

Findings: In this study, the presence of *Helicobacter* spp., the cellular proliferative activity, potential phenotypic alterations, COX-2 and p53 expression in canine spontaneous gastric polyps were investigated. The expression of these molecules was also studied in normal canine gastric mucosa in order to gain further knowledge of the significance of their loss or overexpression in gastric lesions.

Conclusions: The normal expression of almost all the factors evaluated, along with the reduced proliferative activity is strongly suggestive that, in dogs, spontaneous gastric polyps are not only a rare finding but also of benign nature.

Virtual Slides: The virtual slide(s) for this article can be found here: http://www.diagnosticpathology.diagnomx.eu/vs/13000_2014_166

Keywords: Canine gastric polyps, COX-2, CDX2, *Helicobacter* spp, Ki67, p53

Introduction

Gastric polyps (GPs) are considered a heterogeneous entity broadly defined as luminal lesions projecting above the plane of the mucosal surface that can be biologically classified as non-neoplastic and neoplastic lesions. In humans, further classification relies on the evaluation of the histogenesis and neoplastic potential of these lesions [1]. Accordingly, among the non-neoplastic epithelial gastric polyps, three subtypes are recognized: fundic gland polyps; hyperplastic polyps (and variants) and neuroendocrine tumours (NET).

Fundic gland polyps represent the most common type and they are histologically characterized by cistically dilated fundic-type glands lined by flattened parietal cells with a degree of architectural distortion. The surface comprises foveolar type epithelium, which may appear atrophic [1]. The hyperplastic polyps are typically observed in the antrum and histologically consist in hyperplastic, elongated and branching foveolae set in an abundant oedematous and inflamed stroma. Cystic dilation of the pits is almost invariable present in the deeper portions and surface is lined by a single-layer of foveolar-type epithelium [1]. Hyperplastic polyps occur against a background of gastritis and its development is a consequence of an exaggerated mucosal response to injury. They are related to *Helicobacter pylori* (*H. pylori*) infection and some authors concluded that *H. pylori* eradication cause complete regression or significant decrease in its size. It is important to be aware that foci of dysplasia or intramucosal carcinoma can be encountered in these lesions [1,2]. Lastly, NET or the so called "carcinoid" tumours are histologically composed of nests, cords, tubules, and clusters of cells that predominantly interposed between the foveolar basis, without disturbing the overall polyp architecture [3].

Contrary to the documented progression in human colorectal cancer, the association of GPs with the development of gastric cancer is still not fully established but studies emphasize the malignant potential of these

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Amorim et al. Diagnostic Pathology 2014, 9:166
http://www.diagnosticpathology.org/content/9/1/166

lesions. GPs are uncommon in dogs and are occasional incidental findings during endoscopy or postmortem examinations [4]. Sporadic canine GPs are rare and the relevant literature is scarce. Only recently Taulescu et al. [5] described the histopathological features of 15 lesions. Regarding non-neoplastic polypoid growths, the World Health Organization (WHO) for the classification of tumors in domestic animals presents a simplest scheme where only two entities are recognized: the hyperplastic (regenerative) and the inflammatory (benign lymphoid) polyps. The former are histologically identical to the human homonymous lesion and the gold standard of the later is the presence of a normal epithelium covering a granulation tissue core infiltrated by a variety of inflammatory cells, sometimes accompanied by lymphoid aggregates with prominent germinal centers [4].

Several studies trying to involve some markers for malignant transformation in human gastric hyperplastic polyps were performed however, a specific molecule that could identify which polyp will undergo malignant transformation has not yet been found. In the dog, not much is known with this regard.

Cyclooxygenase (COX) is the enzyme that allows synthesis of prostaglandins and other eicosanoids from arachidonic acid. COX-2 isoform plays an important role in gastric cancer development through apoptosis inhibition and increased cell proliferation. Although few studies reported the increased expression of COX-2 in canine cancers no data is available regarding COX-2 expression in canine gastric tissues [6].

Cellular kinetics is thought to participate in the progression towards gastric cancer. Ki-67 antibody is used to measure the proliferation index of a particular tissue assuming that higher Ki-67 reveals tumour cell activity and predicts the further behaviour of a specific pathology [7].

Malignant transformation of human hyperplastic GPs is associated with dysplasia, with p53 playing a crucial role in the process [8]. Overexpression of mutated p53 has been correlated with pathological parameters of tumour aggressiveness and poor prognosis in human gastric carcinoma [9].

According to Correa's model [10], human gastric cancer develops in a multistep process from chronic active gastritis to intestinal metaplasia (IM), dysplasia, and finally, gastric cancer. IM is regarded as precursor lesion, consisting of the transdifferentiation of gastric mucosa to an intestinal phenotype, characterised by the aberrant expression of the homeobox transcription factor CDX2 [11]. Expression of CDX2 in canine GPs has never been characterised.

As uncommon lesions, the molecular properties and neoplastic potential of canine GPs remains an area of interest. The purpose of this study is to describe some molecular features of these lesions in order to better understand their biological behaviour. Additionally, the expression of these molecules in normal gastric mucosa (NGM) was investigated to evaluate the significance of their loss or overexpression in gastric lesions.

Animals & methods

Four normal canine antral gastric mucosa specimens and nine canine antral GPs (Figure 1A), received between 2007 and 2013, were selected from the archives of the Laboratory of Veterinary Pathology, ICBAS-UP (Portugal) and Pathology Department of FVM, Cluj-Napoca (Romania). Tissues were fixed in 10% buffered formalin and paraffin-embedded. Serial consecutive sections 3 μm-thick were made, one for routine histological diagnosis and the others for immunohistochemical studies.

Sections were independently examined by three pathologists. Normal tissues were considered as such according to Prachasilpchain et al. [10] and were negative for the presence of *Helicobacter* spp. (confirmed by modified Giemsa stain and anti-*H. pylori* immunohistochemistry). GPs were classified according to WHO classification for domestic animals diagnostic criteria [3].

For the immunohistochemical study, a panel of antibodies specific for several antigens was applied (Table 1). Novolink Max-Polymer detection system (Novocastra) was used according to the manufacturer's instructions.

Detection of *Helicobacter* spp. was rated as: negative, no organisms, or positive, presence of *Helicobacter* spp. Bacterial density colonization was quantified: +, few (<10/400×); ++, moderate (10-50/400×) and +++, large number of organisms (>50/400×). Bacteria location was recorded: 0 = absent; 1 = mucosal surface and gastric pits; 2 = 1 + lumen of gastric glands (400×) [12]. COX-2 immunoexpression was scored for the percentage of labelled cells (<25%; 25-50%; 51-75%; >75%) and labelling intensity (0, negative; +, weak; ++, moderate; +++, strong). The Ki-67 labelling index was defined as percentage of positive nuclei determined by counting at least 1000 nuclei in the selected fields (×400) [8]. Additionally, immunoreactivity was assessed considering the gastric glands divided in three zones: zone 1 (gastric pit); zone 2 (isthmus/proliferative zone) and zone 3 (gland base) [13]. p53 and CDX2 immunoreactivity were defined as positive when distinct nuclear staining was recognised in at least 10% of the cells [8,14].

Findings

The available clinical data, histological classification of the lesions and main immunohistochemical results, considering the criteria proposed for each antibody, are summarised in Table 2.

Of nine GP lesions, seven were classified as hyperplastic polyps (Figure 1B) and two as inflammatory polyps

Amorim *et al. Diagnostic Pathology* 2014, **9**:166
http://www.diagnosticpathology.org/content/9/1/166

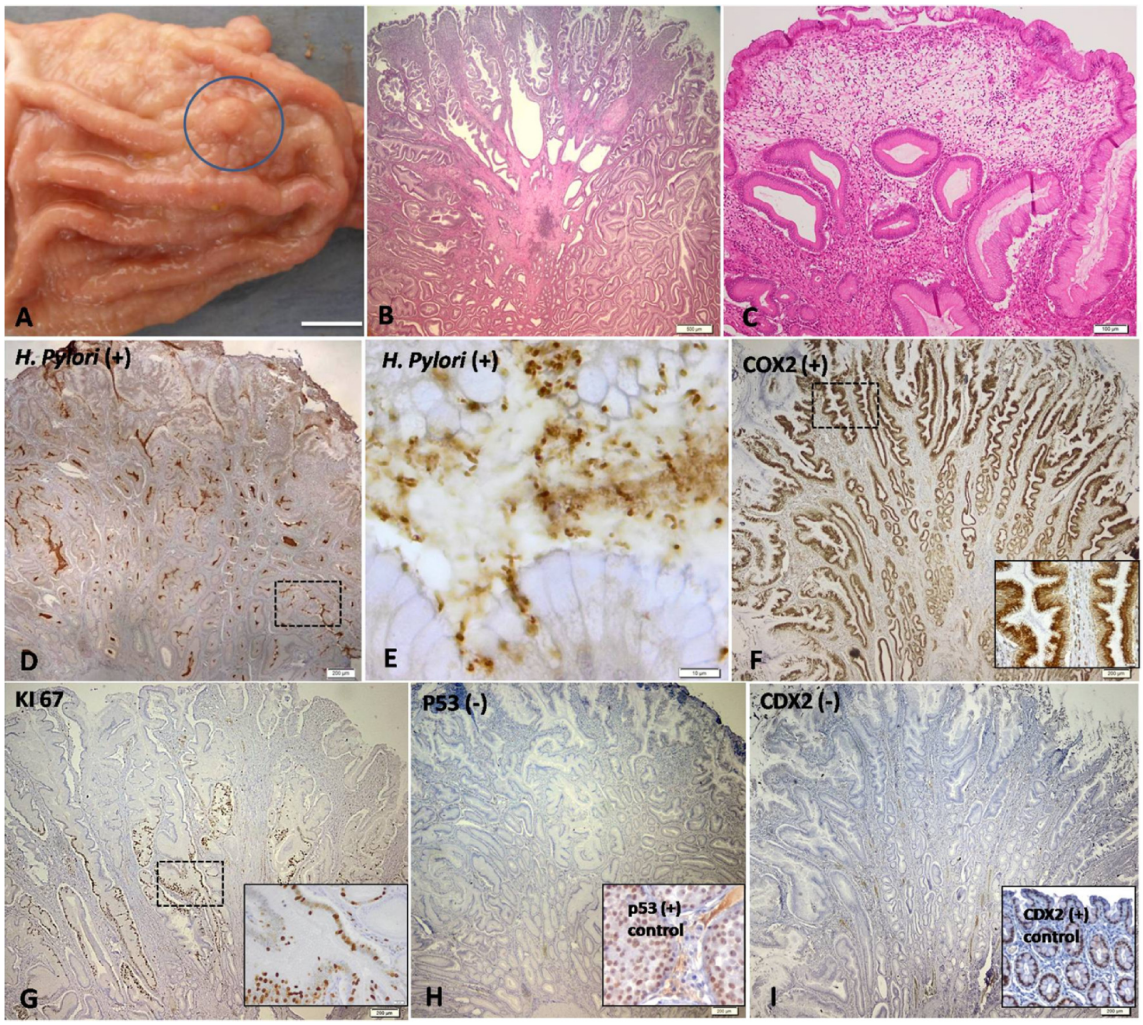


Figure 1 Morphological and immunohistochemical features of the canine gastric polyps. (A) Gross aspect; **(B)** Hyperplastic polyp; **(C)** Inflammatory polyp; **(D) (E)** *H. pylori* immunoexpression. **(F)** COX-2 immunoexpression; **(G)** Ki-67 immunoexpression; **(H)** p53 immunoexpression. Inset: intratubular seminoma (+control) **(I)** CDX2 immunoexpression. Inset: normal intestine (+control). Morphological and immunohistochemical features of the canine gastric polyps. **(A)** Gross aspect of the pyloric gastric mucosa showing a solitary polyp (delimited area) (case 9); **(B)** and **(C)** Microscopic examination of haematoxylin and eosin-stained slides revealing a hyperplastic polyp (case 4) and an inflammatory polyp (case 8). Bar = 500 μm and 100 μm, respectively. **(D)** Photomicrograph demonstrating the positive *H. pylori* expression in a hyperplastic polyp (case 2). Bar = 200 μm. **(E)** Detail of the previous case highlighting the large amount of *Helicobacter* spp. organisms inside the gastric glands. Bar = 10 μm. **(F)** Photomicrograph revealing strong and diffuse COX-2 expression into branched foveolar epithelium of the hyperplastic polyp (case 1). Bar = 200 μm. Inset presenting a detail of the COX-2 expression into foveolar epithelium; **(G)** Immunostaining of Ki-67 in the hyperplastic epithelium (case 3: 32.1%). Bar = 200 μm. Inset showing the detail of KI-67 expression in the isthmus and in the glands base. **(H)** Photomicrograph revealing a negative immunohistochemical reactivity of p53 in the hyperplastic polyp (case 2). Bar = 200 μm. Tissue from canine intratubular seminoma has been used as positive control for p53 (inset). **(I)** Negative CDX2 expression in the hyperplastic polyp (case 1). Bar = 200 μm. Tissue from canine normal intestine has been used as positive control for CDX2 (inset). Immunoperoxidase-diaminobenzidine stain with Mayer's haematoxylin counterstain **(D-I)**.

(Figure 1C). No signs of dysplasia were identified. All GPs revealed *Helicobacter* spp. organisms (Figure 1D), preferentially located in the lumen of the gastric glands (Figure 1E) (Table 2).

Differences on COX-2 immunoexpression between NGM and GPs were minimal (Table 2). Both groups presented strong COX-2 expression in more than 75% of gastric superficial epithelium. Deeply, the gastric glands of NGM showed a weak to moderate COX-2 expression, while the glands of GPs showed strong COX-2 expression (Figure 1F). Nevertheless, GPs presented diffuse COX-2 cytoplasm accumulation in epithelial cells.

Amorim *et al. Diagnostic Pathology* 2014, **9**:166
http://www.diagnosticpathology.org/content/9/1/166

Table 1 Antibodies used for canine gastric tissues immunohistochemistry

Marker	Clone	Supplier	Dilution	Antigen unmasking	Incubation period	Positive control
H. pylori	Polyclonal	Zytomed, Deutschland	1:200	CB/PC	ON	Human gastric mucosa with *H. pylori*
COX-2	SP21	NeoMarkers, USA	1:75	CB/PC	ON	Canine mammary tumour
p53	Anti-p53	Novocastra, UK	1:400	CB/PC	ON	Canine intratubular seminoma
CDX2	AMT28	Novocastra, UK	1:300	CB/PC	ON	Canine normal intestine
KI-67	MIB-1	Dako, Denmark	1:50	CB/PC	ON	Canine high grade lymphoma

CB citrate buffer; PC pressure cooker.
ON overnight.

The proliferative index of GPs was higher (mean value of 24,8%) than NGM (mean value of 20,0%). Ki-67 signal was detected only in the proliferative compartment of NGM whereas in the GPs, this labelling often reached zone 3 (Figure 1G).

Both p53 and CDX2 immunodetection was entirely negative in all cases of canine NGM and GPs (Figure 1H and I).

Discussion & conclusion

Studies regarding COX-2 in human NGM reported lack [15,16], weak [17] or generalised expression [18]. These discrepancies are perhaps related to the primary antibody (monoclonal or polyclonal) and to the scoring system adopted for immunoreactivity evaluation. The consistent expression of COX-2 in NGM could be explained by two hypotheses: (1) COX-2 is constitutively expressed in canine gastric mucosa; or (2) recent studies showed that *H. pylori* induced COX-2 expression in human gastric mucosa and McCarthy et al. [19] concluded that COX-2 expression in antral mucosa was reduced but not eliminated in the epithelium after successful eradication of *H. pylori*. Sung et al. [15] also showed that though eradication of *H. pylori*, there was only a modest reduction of COX-2 in the gastric epithelium, although COX-2 expression in the lamina propria was markedly reduced. Despite these dogs being currently negative for *Helicobacter* spp., it is not possible to rule out a previous infection by these organisms that could justify the persistent expression of COX-2.

The overexpression of COX-2 in the deeper glands of GPs may be explained by the presence of varying amount of *Helicobacter* organisms in this gastric compartment which, similarly to what happens in humans with *H. pylori,* may lead to an increased expression of this enzyme through the production of proinflammatory cytokines [15-17].

Table 2 Available data of the animals, histological classification of the canine gastric tissues and main immunohistochemical findings

Case	Breed	Gender/Age (years)	Histological diagnosis	*Helicobacter* spp.	COX-2		KI-67 (%)	p53	CDX2
					SE	DG			
1	Rottweiler	M/12	Hyperplastic polyp	+++[2]	>75%/+++	>75%/ +++	36,1[2,3]	-	-
2	Irish Setter	F/12	Hyperplastic polyp	+++[2]	>75%/+++	>75%/+++	31,4[2,3]	-	-
3	Poodle	F/12	Hyperplastic polyp	+++[2]	>75%/ +++	>75%/+++	32,1[2,3]	-	-
4	Argentine Mastiff	F/9	Hyperplastic polyp	++[2]	>75%/+++	>75%/ +++	28,9[2,3]	-	-
5	Rottweiler	F/14	Hyperplastic polyp	+[2]	>75%/+++	>75%/+++	30,9[2,3]	-	-
6	Mixed breed	F/16	Hyperplastic polyp	++[2]	>75%/+++	>75%/+++	16,7[2,3]	-	-
7	Poodle	M/13	Inflammatory polyp	++[2]	>75%/+++	>75%/+++	31,5[2,3]	-	-
8	German Shepherd	M/9	Inflammatory polyp	++[2]	>75%/+++	>75%/+++	10,8[2]	-	-
9	Boxer	M/10	Hyperplastic polyp	+[1]	>75%/+++	>75%/+++	4,6[2]	-	-
10	Mixed breed	M/2	NGM	0	>75%/+++	>75%/+	18,3[2]	-	-
11	Poodle cross	F/15	NGM	0	>75%/+++	>75%/++	23,6[2]	-	-
12	Boxer	M/4	NGM	0	>75%/+++	>75%/+	15,6[2]	-	-
13	Mixed breed	M/8	NGM	0	>75%/+++	>75%/++	22,8[2]	-	-

M male, *F* female.
COX-2: SE, surface epithelium; DG, deeper glands. >75% of immunopositive cells, + weak, ++ moderate, +++ strong intensity.
Helicobacter spp.: + few; ++ moderate number; +++ large number of organisms. The bacteria location 0, absent; 1 = on mucosal surface and within gastric pits; 2 = 1+ lumen of the gastric glands; KI-67: zone 2, isthmus and zone 3, gland base; p53 and CDX2: –, negative.

Amorim *et al. Diagnostic Pathology* 2014, **9**:166
http://www.diagnosticpathology.org/content/9/1/166

By definition, a GP is a hyperproliferation of the gastric mucosa, thus its onset is necessarily related to an increase in cell turnover and mitotic activity. Yao et al. reported a proliferative index of 22.2% in human hyperplastic GPs. The slightly difference in the percentages obtained in both studies may be related to the species in question (human vs. dog) and/or with sample size (22 vs. 9 cases). Additionally, increased proliferation of gastric mucosa was associated with *H. pylori* [20] since the infection briefly increases apoptosis of surface and proliferative cells and then expands the proliferative zone to deeper in the gland, presumably as a compensatory response [21].

The proliferative zone in the antral stomach is in the lower third of the typical glands units [21], close to the base of the gastric pits, constituting the site of cellular renewal. This very short area, with scant or no mitoses, is almost unidentifiable in inactive gastric mucosa. In human NGM, Ki-67 signal is present only in the proliferative compartment [13] as such in our study (Table 2). However, in the GPs, this labelling often reached zone 3 (Figure 1G), presumably because in the isthmus there are cells that constantly regenerate others that migrate bidirectionally, up to the mucosal surface and down to the gland base, as they differentiate into mature cells [21].

Carrasco et al. [22] described p53-positive staining in approximately 94.1% of canine gastric carcinomas. Our results reinforce previous human studies in which p53 was only recognised in (pre)neoplastic components [9] rather than non-tumoral gastric mucosa [18] or GPs [8]. Furthermore, Guo et al. [2] found co-expression of both CA19-9 and p53 confined to small areas of atypical epithelial cells, estimated as adenocarcinoma, present in a human hyperplastic foveolar polyp. Additionally, the KI-67 index was higher in these malignant foci when compared with the surrounded gastric mucosa.

Doster et al. [23] described CDX2 expression within foci of metaplastic change, namely goblet cells, present in all the cases of canine gastric adenocarcinomas included in their study. IM is an unusual phenomenon in the dog and up to date there is no description concerning its spontaneous occurrence. In humans, IM is usually induced by continuous irritation of the gastric mucosa and *H. pylori* infection remains an important triggering factor. The molecular pathways involved in canine regulation and function of CDX2 are unknown. However, since the expression of the same transcription factor is associated with the maintenance/development of the intestinal phenotype in both humans and dogs, the hypothesis that CDX2 is similarly regulated in both species is plausible (data not published).

Helicobacter spp. present in cases of canine GPs may develop different strategies of colonization, infection and pathogenicity from those practised by *H. pylori* in humans, eventually justifying the rarity of IM in the stomach of the dog. Therefore, the pathways responsible for the induction of CDX2 expression in dogs may differ from those already described in humans. Further studies are needed to confirm this hypothesis.

Although certain pathological features of polyps correlate with the risk of developing cancer in humans, in dogs it's still not possible to determine which lesions will progress to cancer. In conclusion, the present study suggests that canine sporadic GPs are a rare finding and of benign nature.

Competing interests
None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Authors' contributions
IA, MAT, CC and FG participated in the design of the study. IA and MAT wrote the manuscript. AF and AR carried out the HE and IHC staining. IA, MAT, FG reviewed HE and IHC slides. AMF, CC, CAR and FG drafted the manuscript and revised it critically for important intellectual content. All authors read and approved the final manuscript.

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CHAPTER 5

- General Discussion

In order to understand the possible effects of a bacterium in the stomach of the dog, first it is necessary to know the spectrum of lesions most often found in this host and those in which *Helicobacter* spp. can be simultaneously present.

Thus, the first important step was to obtain a gastric tissue bank collection, in order to initiate and develop the investigation.

The collection of these canine gastric samples was a lengthy and time consuming process. Contrary to what occurs in human medicine, the endoscopy examination is not a routinely used diagnostic technique in veterinary medicine. This can be explained by several reasons: 1) generally, the initial approach of the veterinary clinician when dealing with an animal that presents gastrointestinal signs is to prescribe a treatment. This normally resolves the clinical situation but without a proper and thorough investigation, neither the underlying cause nor the aetiological agent is identified. Only if the animal's clinical signs persist or its condition worsens, the clinician considers adopting other complementary diagnostic techniques such as an abdominal ultrasound or an endoscopy; 2) to perform the endoscopy, the sedation and anaesthesia of the animal is required and in addition to the inherent complications associated with anaesthesia, the price of the examination also increases. Both are comprehensible reasons for the owner to decline this option; 3) the endoscopy procedure requires proper and sophisticated equipment which is not available at all clinics. Therefore, when this examination is indispensable, the animal is referred to a central animal hospital. Since the owner must consider paying further travel expenses, he normally rejects the examination; 4) when all of the aforementioned obstacles are overcome and the endoscopy is performed, another limitation commonly faced is the size of the samples collected during the procedure. This exam in animals is often performed using human paediatric probes, which, depending upon the animal's size, may result in miniscule and less representative samples. Moreover, since this exam is not routinely performed, the clinician has little practice collecting adequate sample sizes.

With all these difficulties, one must consider using samples collected through necropsy exams. However, in most cases, when inspecting the corpse, it may already be in an advanced state of decomposition, inhibiting the collection or further impairing the execution of complementary exams.

Throughout the course of this study and while conducting the various necropsies, we noticed that the presence of gastric *Helicobacter* organisms in dogs that had died more than three hours before the necropsy procedure were very difficult to detect (unpublished observations). We attribute these findings to the low pH levels of the canine stomach that,

along with the varied nature of its gastric content (residual food, grass, soil…), accelerate the decay. As a result, the surface gastric epithelium debris and the *Helicobacter* organisms eventually present are rapidly dragged through the GI tract and lost.

All these hindrances took up a considerable amount of our time. However, we were able to construct a representative collection of canine gastric tissues which include cases of inflammation, mucosal fibrosis and atrophy, proliferative diseases, gastropathies, metaplastic changes, dysplasia and neoplasia. All these entities were thoroughly described in detail in paper I.

As previously mentioned by other groups, inflammation remains the most common canine gastric disease (Happonen *et al.*, 1996; Neiger and Simpson, 2000; Polanco *et al.*, 2011). In fact, gastritis is so commonly found in dogs that animals whose histological pictures are compatible with a normal gastric condition are few and far between.

As is evident in the literature, the prevalence of *Helicobacter* in the canine population is high (Hwang *et al.*, 2002; Moutinho *et al.*, 2007; Shabestari *et al.*, 2008; Van den Bulck *et al.*, 2005; Wiinberg *et al.*, 2005). The same is true for the population within our geographical region (Paper III). As such, the combination of gastritis plus *Helicobacter* was also a typically encountered scenario.

In the majority of cases examined, the bacteria were accompanied by mild to moderate chronic gastritis, especially of the lymphoplasmacytic type. Nevertheless, and like other groups, we were not able to determine a significant statistical relationship between gastritis and the presence of *Helicobacter* ($p>0.05$) (Eaton *et al.*, 1996; Hermanns *et al.*, 1995; Polanco *et al.*, 2011; Shabestari *et al.*, 2008). There was, however, a significant correlation between the presence of the bacteria and both mild to moderate intraepithelial lymphocyte infiltration and mild to moderate epithelial injury in the canine stomach ($p<0.05$) (Paper III).

H. pylori is well accepted as an important human pathogen and is believed to be the primary cause of chronic gastritis, gastric and duodenal ulceration, and even gastric carcinoma and MALT lymphoma. On the other hand, the pathogenic significance of gastric *Helicobacter* species in dogs is still controversial. Considering the role that glycan structures play as receptors for *H. pylori* adhesins in human gastric mucosa (Magalhães and Reis, 2010), the knowledge of the glycan structures expressed by canine gastric cells constitutes an important basis for understanding the relevance of bacterial epithelial adhesion in the outcome of *Helicobacter spp.* canine infection.

In the human normal gastric mucosa, and depending on the secretor status of the individual, Le^a and Le^b are highly expressed in the superficial foveolar epithelium (Lopez-Ferrer *et al.*, 2000; Mollicone *et al.*, 1985), where MUC5AC mucin is coexpressed (Teixeira

et al., 2002). The expression of Lewis type 2 antigens is generally restricted to the deeper part of the gastric glands, where MUC6 mucin is co-expressed (De Bolos *et al.*, 1995; Moran, 2008).

The expression pattern of type 1 and type 2 Lewis antigens in the canine stomach revealed a different distribution when compared to the human gastric mucosa (paper II) (Amorim *et al.*, 2014a). The absence of type 1 Lewis antigens expression is explained by the lack of expression of enzymes with α1,4 fucosyltranferase activity. Additionally, extensive expression of type 2 Lewis structures and A antigen, both in the surface and glandular epithelium, was observed (Amorim *et al.*, 2014a).

In humans, the expression of both sialyl-Le^a and sialyl-Le^x is associated with *H. pylori* infection (Mahdavi *et al.*, 2002; Ota *et al.*, 1998). The increased expression of sialylated structures in response to *H. pylori* infection is not a human-specific phenomenon and has been described in different animal models. Experimentally infected Rhesus Monkeys also showed increased expression of sialyl-Le^a and sialyl-Le^x in surface epithelial cells (Linden *et al.*, 2008; Mahdavi *et al.*, 2002).Similarly, it has been shown that sialyl-Le^x expression is absent in healthy gastric epithelial cells of Mongolian gerbils but increased in infected and inflamed gastric mucosa (Ohno *et al.*, 2011). In the current study, we found sialyl-Le^x expression in the body and antrum region of 50% and 10% of the dogs, respectively. Despite the gastric tissues used in our study were histologically classified as normal and were negative for H*elicobacter spp*., is not possible to rule out a previous infection by these organisms that could have induced the sialyl-Le^x expression observed.

According to the results of the *in vitro* binding assays, *H. heilmannii s.s.* was the species of NHPHs group that showed the highest adhesion to the canine gastric mucosa (Amorim *et al.*, 2014a). In agreement, *H. heilmannii s.s.* was also the NHPH species more prevalent in spontaneous infected dogs in our geographical location (paper III). This clearly contrasts with other reports which describe *H. bizzozeronni* as the NHPH most frequently found in the canine stomach (Priestnall *et al.*, 2004; Van den Bulck *et al.*, 2005; Wiinberg *et al.*, 2005).

In addition, the *H. heilmannii s.s.* adhesion value obtained *in vitro* was higher in the antrum than in the body compartment of the canine gastric mucosa (Amorim *et al.*, 2014a). This finding was further corroborated by our epidemiological data which identified this as the most prevailing species in the antral portion of the stomach (paper III).

The other NHPHs presented a lower *in vitro* adhesion rate compared to *H. heilmannii* s.s.. In descending order, *H. felis* was the second most adherent bacteria, followed by *H. bizozzeronii* and then *H. salomonis* and this tendency was observed in both stomach compartments (Amorim *et al.*, 2014a).

The second most prevalent species found in the Portuguese dogs was *H. salomonis* (paper III). Interestingly, this bacterium was most frequent in the body gastric region and it was found mainly in association with other NHPH (paper III). In contrast, *H. salomonis* has only been sporadically detected in dogs (Haesebrouck *et al.*, 2009) and Van den Bulck *et al.* (2005) did not find any cases of single infection by this species (Van den Bulck *et al.*, 2005). Accordingly, in the *in vitro* binding assays this was the species that adhered less to the canine stomach (Amorim *et al.*, 2014a).

Given these results and the proportion of dogs detected in our study showing the *H. heilmannii s.s* and *H. salomonis* bacterial combination, it seems plausible that in the dog, direct competition between at least these two species may occur in order to achieve the successful colonization of the gastric niche. Furthermore, the high percentage of mixed infections comprising *H. salomonis* may suggest that the colonization capacity of this specific species in the gastric context may be enhanced when associated with other NHPH (Paper III). However, further studies are needed in order to clarify these hypotheses.

Mixed infections are a common finding in canine NHPH infection (Haesebrouck *et al.*, 2009; Van den Bulck *et al.*, 2005; Wiinberg *et al.*, 2005). Wiinberg and colleagues found co-infection in 16.7% of the dogs included in their study. With the exception of one animal infected with *H. felis* and *H. bizzozeronnii*, the majority were infected with a combination of *H. heilmannii* and *H. bizzozeronii* (Wiinberg *et al.*, 2005). It is important to be aware that in this "*H. heilmannii*" nomenclature of Wiinberg and colleagues' publication, all the other NHPH different from *H. felis* and *H. bizzozeronnii* were included. Additionally, *H. bizzozeronni* was not only the most prevalent species, but it was also detected in 50% of the dogs studied with mixed infections (Van den Bulck *et al.*, 2005).

It should be taken into account that the epidemiological studies available in the literature were conducted in different countries, such as Finland and Denmark (Northern Europe); Switzerland and Belgium (West-Central Europe) and the USA. Thus, it is reasonable to assume that geographic variations account for the prevalence of a specific *Helicobacter* species in the dog stomach. Similarly, *H. pylori* strains from different geographic areas are associated with clear phylogeographic variation; therefore, comparisons of the genetics of *H. pylori* can serve as a marker of the migration of human populations (Yamaoka *et al.*, 2008).

According to the *in vitro* results, the *H. pylori* control strain, 17875/Le^{b}, which is a BabA positive strain, adhered less than *H. heilmannii s.s. (Amorim et al., 2014a)*. This can be explained by the fact that the dog does not have Lewis type 1 antigens. The 17875*babA1A2*, which is a SabA positive strain, adhered significantly more in the body region because in this area the dog has sialylated antigens, namely sialyl-Le^{x}. Nevertheless, *H. pylori* is rarely found in the dogs' stomach (Buczolits *et al.*, 2003; Chung *et al.*, 2014; Ekman *et al.*, 2013).

Perhaps the dog has other mechanisms that prevent the colonization.

One must be aware that the two *H. pylori* strains that were used are model strains. They are highly adherent and therefore, the higher value achieved *in vitro*, particularly by 17875*babA1A2* was not so surprising. However, probably due to competitive factors between bacteria and/or different immunological responses of the host, *in vivo H. pylori* often fails to colonize canine gastric mucosa. Unfortunately, in our study aimed at determining *Helicobacter* prevalence, we did not include a specific primer for *H. pylori* PCR identification and as such, we are unable to compare the *in vitro* binding results with those of the spontaneous *in vivo* infection.

Moreover, in the *in vitro* binding assays, the mean adhesion values (bacteria/μm^2) obtained with *H. pylori* strains and NHPH were remarkably different. This may suggest that *H. pylori* adheres more to gastric epithelium than NHPH, as previously documented (Schreiber *et al.*, 2004; Schreiber *et al.*, 1999). On the other hand, it has been suggested that *H. pylori* may present an inter-epithelial location and that few bacteria are able to invade the cell membrane, being internalized in epithelial or immune cells (Dubois and Boren, 2007). A previous study in Beagle dogs also documented the presence os spiral shaped bacteria inside macrophages (Lanzoni *et al.*, 2011). According to our observations, the presence of spiral-shaped bacteria in parietal intracytoplasmic location is a common finding in the canine stomach and this phenomena seems to be an exclusive feature of NHPH (Geyer *et al.*, 1993; Happonen *et al.*, 1996; Hermanns *et al.*, 1995; Lanzoni *et al.*, 2011; Scanziani *et al.*, 2001). Even when the superficial mucus seems depleted and there are no signs of *Helicobacter spp.* lying in the mucus or within the gastric glands, ultimately, the immunohistochemistry permitted the detection of the bacteria in this subcellular location without displaying visible pathological changes to these cells. The significance of this finding is yet to be explored. The *in vitro* binding studies performed only assess the organisms present at the cell surface and do not fully consider those in intracellular locations. As such, further comprehensive and ultrastructural studies are warranted to unravel the value of this observation in the canine stomach.

During our study, we also intended to characterize the expression pattern of some mucins, namely MUC1, MUC2, MUC5AC and MUC6, in order to obtain broader insight of the glycoproteins' distribution in the canine stomach. However, we did not succeed in this task because the majority of the antibodies are produced for human use only and due to the mucins' interspecies variability, reactivity with canine tissues was not obtained.

The discrepancy amongst our results obtained both *in vitro* and from spontaneously infected dogs demonstrate that, like any other host-pathogen interaction, other more complex factors play a role in the colonization and the establishment of the infection which were not even approached in the present investigation. Nevertheless, when it concerns the

adhesion process, the mechanisms recruited certainly differ between *H. pylori* and NHPH. The fact that bacteria so closely phylogenetically related can act so differently in the same context is curious.

In the present investigation, we were able to show that, despite the high density of *Helicobacter* colonization in canine gastric polyps, the very molecular features of these lesions reinforce their benign character (paper IV) (Amorim *et al.*, 2014b).

The prevalence of *H. pylori* in human hyperplastic gastric polyps is 95% to 100% (Ohkusa *et al.*, 1998; Saito *et al.,* 1992). Herein, *Helicobacter spp.* organisms were also identified in all the cases of GP. Although some studies reported a relationship between *Helicobacter spp.* and gastric polyps (Taulescu *et al.*, 2014), the pathogenic effects of these bacteria in dogs is debatable. In our study, the exact identification of the *Helicobacter* species present in these canine lesions was not possible, but we recognize that this would be a worthwhile endeavour.

Some human immunohistochemical studies demonstrated overexpression of COX-2 in gastric lesions, such as polyps (Bamba *et al.*, 2003), intestinal metaplasia (Sung *et al.*, 2000) and carcinomas (Ohno *et al.*, 2001). This expression tends to increase along with the severity of the lesion (Bamba *et al.*, 2003; Ohno *et al.*, 2001). In our series of GPs we detected an overexpression of COX-2 in gastric epithelial cells which presented a most diffuse and usual cytoplasm accumulation. This can be explained by the presence of varying amount of *Helicobacter spp*. organisms, which similarly to what happens in humans with *H. pylori*, may lead to an increased expression of this enzyme through the production of proinflammatory cytokines. The fact that these bacteria are located preferentially within the gastric glands of GP may also explain the higher intensity of COX-2 expression in this gastric compartment, in contrast to what is seen in normal mucosa.

A higher proliferation index was found in canine GP when compared to that counted in normal gastric mucosa (Amorim *et al.*, 2014b). According to its definition, a GP is a hyperproliferation or overgrowth of the gastric mucosa towards the lumen thus, its onset is necessarily related to an increase in cell turnover and mitotic activity. Yao *et al.* (2002) recorded the proliferative index in 22 human hyperplastic GPs, reaching an average value of 22.2%. The slight difference in the percentages obtained in both studies may be related to the species in question (human vs dog) and/or with the sample size (22 vs 9 cases) or even with the biological stage of the GP. Assuming that polyps begin to grow up to a certain point, it is likely that those who are still in development show a higher proliferative index in comparison with the older ones, whose growth is stabilizing or has already stagnated.

In humans, previous reports make reference to the fact that an increased proliferation of the gastric mucosa could be associated with the presence of *H. pylori* (Kim *et al.*, 2004). *H.*

pylori infection initially and briefly increases apoptosis of surface and proliferative cells and then expands the proliferative zone to deeper in the gland, presumably as a compensatory response (Mills and Shivdasani, 2011). Even so, in our study, it was not possible to establish a correlation between the density of *Helicobacter* colonization and the proliferative index of canine polypoid lesions.

Malignant transformation of human gastric hyperplastic polyps is assumed to be associated with dysplasia, with p53 playing a crucial role in the process (Yao *et al.*, 2002; Zea-Iriarte *et al.*, 1995). P53 reactivity was also observed in human hyperplastic polyps but limited to adenomatous foci suggesting that its overexpression occurs in dysplastic epithelium of precancerous gastric lesions (Lauwers *et al.*, 1993). P53 protein immunodetection was entirely negative in all the cases of GPs. Our results go towards some previously reported in humans, in which p53 was only recognized in (pre)neoplastic components (dysplasia and carcinoma) rather than non-tumoral gastric mucosa (Sereno *et al.*, 2006), gastric hyperplastic areas (Zea-Iriarte *et al.*, 1996) or gastric hyperplastic polyps (Yao *et al.*, 2002).

Several metaplastic changes are pre-neoplastic lesions and predispose individuals to develop intestinal-type cancer. In humans, IM is usually triggered by the continuous irritation of the gastric mucosa and *H. pylori* infection is known to be one of the most important triggering factors. Up to date there is no description concerning its spontaneous occurrence in canine stomach. Furthermore, our results describe the lack of IM in canine GPs. Possibly, the *Helicobacter* species present in these cases of canine GP develop different strategies of colonization, infection and pathogenicity from those practiced by *H. pylori* in humans, eventually justifying the rarity of IM in the stomach of this animal species and therefore, the lack of CDX2 immunoreactivity.

Another task we are developing but unfortunately have not been able to finalize at this stage, is the research of somatic mutations in canine gastric cancer. It is well accepted that the E-cad plays an important role as an invasion suppressor gene/protein, since its loss of expression, abnormal function or both, leads to an increased ability of cells to invade neighbouring tissues, as verified in cancer. Indeed, genetic or epigenetic alterations in *CDH1* E-cad-encoding gene or alterations in its protein expression often result in tissue disorder, cellular differentiation, increased invasiveness of tumour cells and ultimately, in metastasis (Paredes *et al.*, 2012). In human gastric cancer, downregulation of E-cad is a feature of diffuse/isolated cell carcinomas and occurs in the large majority of cases. Similar abnormalities on E-cad expression were observed in a representative series of canine CG (paper I). E-cad absence or aberrant expression may be caused, in sporadic diffuse/mixed gastric cancer cases, by somatic mutations in the *CDH1* gene. In hereditary forms of diffuse gastric cancer first described by Guilford in 1998, *CDH1* gene germline alterations are causing events. Nothing is known in this regard when it comes to canine gastric cancer.

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- Summary and Conclusions

Since the discovery of *H. pylori* in the early eighties and its association with gastric pathology, research on the *Helicobacter* genus has increased tremendously. In addition to *H. pylori*, other helicobacters commonly denominated non-*H. pylori Helicobacters* are found to colonize the human stomach. These organisms have also been associated with gastric disease in humans and the important role of the dog in the transmission of NHPH to humans has been highlighted.

In this investigation we characterize the epidemiological, pathological and molecular features of the spontaneous gastric infection with different NHPH in canine stomach and evaluated the relevance of particular glycan structures in *Helicobacter spp.* adhesion to canine gastric mucosa. To achieve this goal, a logical succession of steps were taken. These are thoroughly detailed and organized within this manuscript, as follows:

- In the **first chapter** the aims and outline of this thesis are described.

An overview of the most common canine gastric lesions was performed and whenever appropriate, these were compared with the equivalent human disorders. Additionally, recognized human gastric histopathological changes, namely glassy cells change, globoid dysplasia and other histological variants of gastric cancer, were described for the first time in the canine stomach. In this chapter, we also proposed that the current histological classification system for the classification of canine gastric cancer should be revisited.

- In the **second chapter** we evaluated and described for the first time the glycosylation profile of both the body and antral compartments of the canine stomach. Additionally, the *in vitro* binding capacity of FITC-labelled *H. pylori* and NHPH in cases representative of the canine gastric glycosylation pattern was tested. Firstly, we demonstrated that the canine gastric mucosa lacks expression of type 1 Lewis antigens and presents a broad expression of type 2 structures and A antigen, both in the surface and glandular epithelium. The absence of type 1 Lewis antigens was explained by the lack of expression of enzymes with α1,4 fucosyltranferase activity. Lewis X (Le^x) revealed a variable expression in the body, whereas expression was detected in the antrum of all animals. Expression of sialylated Le^x, involved in SabA-mediated adherence of *H. pylori*, was mainly observed in the body. Regarding the canine antral mucosa, *H. heilmannii s.s*. presented the highest adhesion score whereas in the body region the SabA-positive *H. pylori* strain was the strain that adhered the most.

- In the **third chapter** we evaluated the prevalence of NHPH in the stomach of dogs in our geographical location and assessed the histopathological changes associated. We concluded that the number of canine stomachs that met the criteria to be considered histologically normal was very low. We attested that gastritis in dogs is extremely common and that, within our geographical location, the presence of *Helicobacter* in this host reaches elevated levels. The prevailing species was *H. heilmannii s.s.,* and *H. salomonis* was found to be a species with a relevant participation in mixed infections. Additionally, the immunohistochemistry was considered a suitable technique for *Helicobacter* detection in the canine stomach.

- In the **fourth chapter** we describe some molecular features of spontaneous canine gastric polyps in order to better understand their biological behaviour. Despite the high density of colonization by *Helicobacter* organisms, we concluded that these lesions do not exhibit pertinent histomorphological and molecular changes, which favour its benign nature and behaviour.

- In the remaining chapter the main findings of this thesis are discussed and further directions for future research on dog-associated NHPH are provided.

Overall our work provides an important contribution to the understanding of the gastric pathological disorders that can affect the dog. Moreover, our results have extended beyond the current knowledge of normal and pathological conditions encompassing canine gastric mucosa, namely the glycosylation profile and the molecular mechanisms underlying dog-related *Helicobacter* species adhesion.

In conclusion, our results support that the differences in gastric mucosa glycosylation profile **play a role in the host adaptation of the gastric *Helicobacter* species** and **indicate that adhesion of NHPH involves different mechanisms**, probably mediated by proteins with alternative receptor specificity yet to be characterized. Furthermore, despite the high prevalence and worldwide distribution of NHPH, **geographic variations partially account for the prevalence of a specific *Helicobacter* species in the dog stomach**.

- Future Work and Perspectives

Most of the research in gastric pathology has been focussed on *H. pylori*. However, the gastric NHPH species are of zoonotic significance and few attempts have been made to unravel the pathogenic mechanisms of canine helicobacters. Thus, in the future, **we would like to investigate the strategies of adhesion of dog-associated NHPH in the human and canine gastric mucosa** in order to fully understand the zoonotic hazard originating from dogs.

The majority of dogs infected with NHPH present limited inflammatory response and absence of clinical signs. This evidence raises the question if these organisms are commensal rather than pathogenic in the canine stomach. Nevertheless, the presence of these bacteria in canine stomach in conjunction with other gastric alterations and pathologies, have been observed by our group and others. However, in the vast majority of the existing studies, the NHPH were not identified at the species level, which complicates the interpretation of these findings. Thus, a first issue that we would like to address is: **to determine if the interactions with the canine gastric mucosa vary between and within different NHPH species**. For that, it would be interesting to use canine cells lines and tissues and incubate them with different strains belonging to different gastric *Helicobacter* species. This would allow us to obtain better insights about the possible pathogenic significance of these NHPH species for dogs.

In the present study we also concluded that *H. pylori* and dog-associated NHPH present different abilities to adhere to canine gastric mucosa and that these differences play a role in the host adaptation. Thus, it is reasonable to speculate that **the capacity to adhere and to colonize the human gastric mucosa may also vary between different gastric *Helicobacter* species**. To further confirm this hypothesis, it would be interesting to evaluate the ability of different *Helicobacter* strains belonging to different NHPH species to adhere to the human gastric mucosa, recurring to well established *in vitro* models.

Another important goal is **to identify the glycan receptors involved in the adhesion of NHPH to gastric mucosa**. To achieve this, it would be a major challenge to use glycan microarray analysis as it is a powerful high-throughput tool. This would provide important information regarding *Helicobacter*-glycan adhesion and would give significant insights into the binding specificity and biological activity of the *Helicobacte*r spp..

Last but not least… Now that I'm about to become a *philosophy doctor*, I wish one day I could become a **veterinary pathologist**.

Appendix

Histol Histopathol (2014) 29: 65-75

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Histology and Histopathology

Cellular and Molecular Biology

Histopathological features of canine spontaneous non-neoplastic gastric polyps - a retrospective study of 15 cases

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Summary. Fifteen cases of canine gastric polyps, collected over a 4-year period, were investigated using gross inspection, histological procedures and immunohistochemical techniques for *Helicobacter* infection. No breed or sex predisposition was found for gastric polyps, although they occurred mainly in elderly animals. There were 9 pedunculated and 6 sessile polypoid growths, between 5 to 30 mm in diameter developed mainly in the pyloric region of the stomach. The most common type of gastric polyps was the hyperplastic one. The inflammatory type was identified in three cases. Foci of AB/PAS Goblet positive cells resembling intestinal metaplasia, mild dysplasia of gastric epithelium, well delimited calcified areas, islands of osteoid matrix and nematodes were present in some of these lesions. Histological examination of the adjacent gastric polyp (surrounding gastric mucosa) revealed a severe chronic inflammation in 13 cases and a high grade of *Helicobacter* species colonization in all cases, but Kendall test analysis showed no correlation between *Helicobacter* spp. colonization degree and gastritis scores (τ=0289; p=0.204). A significant correlation was found between *Helicobacter* spp. location and gastritis scores (τ=0.497; p=0.035). Immunohistochemistry performed with a polyclonal antibody confirmed *Helicobacter* spp. infection in all cases. Based on their morphology, *Helicobacter pylori* - like organisms were described in 3 of 15 cases. No high degree of dysplasia nor neoplasia were identified in these lesions. The etiology and pathogenesis of gastric polyps in dogs are still unknown, although a severe chronic antral gastritis may be a predisposing condition for development of gastric polyps in dogs.

Key words: Chronic gastritis, Dogs, Gastric polyps, *Helicobacter* species infection, Immunohistochemistry.

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Introduction

Polypoid growths arise from mucosa surfaces as the result of either hyperplasia or neoplasia (Head et al., 2003). In humans, gastric polyps (GPs) are incidentally detected in 2-3% of upper gastrointestinal endoscopic examinations (Silverstein and Tytgat, 1997). The majority of these polyps (85-90%) are hyperplastic, located at the junction of fundic and pyloric mucosa (Silverstein and Tytgat, 1997; Jain and Chetty, 2009). Multiple hyperplastic polyps are commonly found in about 20% of human patients (Abraham et al., 2001). In humans GPs are associated with inflammatory mucosal

disease, most frequently chronic active *H. pylori* gastritis. Both hyperplastic and inflammatory GP can be found in chronic *H. pylori* gastritis, although these histological patterns do not have premalignant significance (Abraham et al., 2001). Adenomas arising in atrophic gastritis with intestinal metaplasia also occur and may have malignant potential (Kamiya et al., 1982).

Gastric polyps occur rarely in dogs (Van der Gaag, 1988; Diana et al., 2009, Kuan et al., 2009), cats (Smith et al., 2009), horses (Moorse and Richardson, 1988) and cattle (Yamaguchi et al., 2004). Benign GPs are uncommon incidental findings in dogs that can be observed during gastric endoscopy or necropsy procedures. These polyps are frequently found in the pyloric region, rarely causing pyloric stenosis (Diana et al., 2009; Kuan et al., 2009). Grossly, GP can be solitary or multiple appearing as bulging sessile or pedunculated formations, with an arborescent surface, measuring about 7-60 mm in size (Happe et al., 1977; Diana et al., 2009). Histologically, polypoid non-neoplastic growths of dogs are hyperplastic (regenerative) and inflammatory (benign lymphoid), while neoplastic ones are adenoma and carcinoma (Head et al., 2003). Hyperplastic and adenomatous GP types have been described in dogs (Gualtieri et al., 1996; Diana et al., 2009; Kuan et al., 2009). Etiology of GP in dogs is still unknown, but some authors suppose a possible hereditary predisposition in French bulldog (Gualtieri et al., 1996; Kuan et al., 2009).

Several species of *Helicobacter* are identified in dogs: *H. heilmannii*, *H. bizzozeronii*, *H. felis*, *H. salomonis* (Eaton et al., 1996; Happonen et al., 1998; Jalava et al., 1998, Lanzoni et al., 2011) and *H. canis* (Ekman et al., 2013). On the other hand, *H. pylori* has occasionally been identified in the canine stomach (Buczolitis et al., 2003; Ogawa et al., 2005). Natural infection by *Helicobacter* spp. is associated with lymphoid nodular hyperplasia, diffuse inflammatory infiltrate with mononuclear cells, glandular degeneration, mucosal atrophy and fibrosis. No correlation has been found between the level of bacterial colonization and the severity of gastritis (Lee et al., 1992; Hermanns et al., 1995; Happonen et al., 1996). However, recent reports showed that high density of colonization might be connected with the clinical signs (Hwang et al., 2002). Additionally, nutritional, environmental factors and the individual immune response might play a role in the induction of chronic gastritis.

Several genus of nematodes, namely *Ollulanus, Gnathostoma, Toxocara canis* and *Physaloptera* spp. are present in dogs but rarely cause chronic gastritis. The tissue reaction to these larvae results in epithelial hyperplasia and polyp-like growths of the antral mucosa (McGavin and Zachary, 2007).

There are few publications regarding the histomorphological features and etiopathogenesis of GP in dogs. The aim of this retrospective study was to determine the incidence of GP in canine necropsies and to describe their gross and histological features. The evaluation of the surrounding gastric mucosa was also performed in order to determine its relationship with canine gastric *Helicobacter* infection. Additionally, the search for a pathophysiological link between the severity of gastritis and GP development was intended.

Materials and methods

Animals

A total of 643 necropsied dogs submitted to the Pathology Department, Faculty of Veterinary Medicine from Cluj-Napoca (Romania), from 2007 until 2010 were investigated. Among these, 73 animals presented different gastric lesions and GP were identified in 15 of these cases. All the cases were evaluated concerning the animal clinical history or signs related to gastrointestinal disease.

Necropsy

Complete necropsy procedure was performed in all dogs. The stomach was opened along the lesser curvature and inspected for gross lesions. Polypoid masses were reviewed for location, number, size, shape, colour and associated lesions. Samples for histology were collected from various tissues, according to the gross findings. Full-thickness sections of GP and surrounding gastric wall were also selected.

Histology

Samples were fixed in 10% buffered formalin (pH=7) for 24 hours, embedded in paraffin wax, cut into 3-5 μm sections and stained with hematoxylin and eosin (HE), Masson's trichrome (MT), Alcian Blue/Periodic Acid Schiff (AB/PAS) and May-Grunwald Giemsa for *H. pylori* stains (code 26114, EMS Industry Road Hatfield, PA).

Immunohistochemistry (IHC)

For the immunohistochemical study, antigen retrieval was performed on dewaxed sections in a pressure cooker in 10 mMol/l sodium citrate buffer (pH 6.0) for 3 min. Slides were cooled for 10 min at room temperature and rinsed twice in phosphate buffered saline (PBS) for 5 min. After blocking endogenous peroxidase activity with hydrogen peroxide 3% in phosphate-buffered saline, slides were incubated in goat serum (code X0907, Dako Denmark) in PBS to reduce background staining. Sections were then incubated with the primary antibody rabbit polyclonal anti *H. pylori* (code B0471, Dako Denmark) dilution 1:10, overnight at 4°C. Sections were rinsed with PBS between each step of the procedure. The secondary antibody (labeled

streptavidine biotine) was applied for 30 min. Labelling was 'visualized' by incubation of the sections with a freshly prepared solution of 3,3'-diamino-benzidine (DAB) (LSAB System-HRP kit (code K0679, Dako Denmark). Finally, sections were lightly counterstained with haematoxylin, dehydrated and mounted.

Gastric biopsies from human patients infected with *Helicobacter pylori* confirmed by PCR were used as positive controls, while in negative controls the primary antibody was omitted and replaced by mouse IgG1 (code X0931, Dako Denmark).

Sample evaluation

The slides were evaluated in two steps. First, the sections were independently examined by three pathologists and, when there was a divergence of opinion, an agreed diagnosis was reached by using a multihead microscope (Olympus BX51 microscope with Olympus SP 350 digital camera). Cell B" basic imaging software (Olympus) was used for semi automat counting of cells, glands and bacteria.

Histological classification of GP was done according to WHO classification of Tumors of the Alimentary System of Domestic Animals (Head et al., 2003). Additionally, other histological features of the polyps, such as preneoplastic criteria (glandular atrophy, intestinal metaplasia and epithelial dysplasia) were evaluated in each case.

HE and TM stained sections from adjacent gastric mucosa were examined to establish the type and intensity of inflammation according to the Updated Sydney System classification for human gastritis (Dixon et al., 1996). Quantification of inflammation was done according to grading system: 0 (*normal*), containing 0 to 10 inflammatory cells per high-power field (x400) with no lymphoid follicular aggregates and normal mucosal epithelium; 1 (*mild gastritis*), containing 10 to 50 inflammatory cells/ (x400) with fewer than two lymphoid follicles per low-power field (x40) and normal mucosal epithelium; 2 (*moderate gastritis*), containing 10 to 50 or more inflammatory cells/ (x400) with more than two follicles per low-power field and mild gastric epithelial changes; 3 (*severe gastritis*), containing more than 50 inflammatory cells/(x400) and marked epithelial changes. Epithelial changes included individual cell necrosis, basophilic cytoplasm, and glandular dilation (Handt et al., 1995). An increased number of mononuclear cells was diagnosed as chronic gastritis. Sparse lymphocyte aggregates without inflammatory cellular infiltrate in the lamina propria were considered normal. The terms superficial gastritis and diffuse gastritis were used to indicate the distribution of inflammatory cells in the gastric mucosa.

Giemsa for *H. pylori* stained slides were used to quantify the colonization density of the *Helicobacter* spp. in adjacent gastric mucosa: 0, no organisms seen; 1, few organisms (<10/ x400); 2, moderate numbers of organisms (10 to 50/ x400); 3, large numbers of organisms (>50/ x400) (Prachasilpchai et al., 2007). Histological evaluation of *Helicobacter* spp. location at high power field (x400) was done: grade 0=none; grade 1=on mucosal surface and in gastric pits; grade 2=1+in gastric glands; grade 3=1+2+in parietal cells (Happonen, 1999).

IHC evaluation was scored as follows: negative, no evidence of *Helicobacter* spp. or positive, presence of *Helicobacter* spp. antigen indicated by a brown colour.

Statistical analysis

For statistical analyses, SPSS software, version 16 and correlation (Kendall) tests for nonparametric measures ($p<0.05$) was used. Kendall test was also applied to determine the correlation between *Helicobacter* spp. degree/location with gastric inflammatory scores.

Results

The anamnesis data and the main findings of the 643 necropsied dogs are summarized in table 1. Macroscopic gastric lesions were observed in 73 dogs (11.3%) and, among these, 15 had GP (20.5%). Gastric polypoid masses were found in elderly animals of various breeds, between 9 and 17 years of age (average 12.5 years). Cases were equally distributed between sexes (8 females and 7 males). In addition to the signs related to the main cause of death or euthanasia, evaluation of the history of some patients revealed chronic vomiting in seven cases (1, 3, 4, 5, 6, 8 and 12, representing 46.6% of dogs with GP), and gastric dilation in cases 8 and 11 (13.3% of dogs with GP). Weight loss was reported in cases 1, 2, 5, 8 and 12 (affecting 33.3% of dogs with GP) and this could be explained by the main pathologic conditions, i.e. cirrhosis, chronic renal failure and metastatic cancer.

Gross appearance and topographical distribution of the gastric polyps

Gastric examination revealed pedunculated (9 cases) and sessile (6 cases) polypoid growths with white-grayish or white-reddish colour, firm, smooth or irregular surface, and well vascularized. Solitary polyps were found in 7 cases (Fig. 1a), while multiple gastric polyps (between two to nine masses) (Fig. 1b,c) were revealed in 8 dogs. The size of these lesions varied from 0.5 to 3.0 cm in diameter.

Almost all gastric polypoid masses, solitary or multiple, were found in the pyloric area of stomach (Table 1). No GPs were found in the cardiac gland region, or in the fundic region.

In 10 out of 15 cases, chronic hypertrophic gastritis was grossly evident in the pyloric region. Two of these cases had gastric dilation due to pyloric stenosis (8 and 11).

Pathological findings of canine gastric polyps

Gastric polyp's histopathology

Histopathologic examination confirmed benign gastric polyps in all the cases with antral polypoid growths (12 hyperplastic, 3 inflammatory) (Table 2).

Twelve cases (78.5%) were diagnosed as hyperplastic polyps based on the following features: marked elongation of the gastric pits with cystic dilation, branching of both foveolae and stromal core (reactive mesenchymal cells - myofibroblasts) of the lamina propria (Fig. 2a), a moderate mixed inflammatory infiltrate with plasma cells, lymphocytes, eosinophils, macrophages, and scattered neutrophils (Fig. 2b). Multiple smooth muscle fibers originating from the *muscularis mucosae* were identified in the lamina propria between the gastric pits and glands. The foveolae were lined by hyperplastic gastric epithelium (Fig. 2c). Both the epithelium and the mesenchymal stromal cells showed marked regenerative changes. In three cases, minimal change of foveolar epithelium was observed along with goblet cell metaplasia (Fig. 2d), further confirmed by AB/PAS stain (Fig. 2e). In one hyperplastic GP multiple areas of acelular and hyaline aspect compatible with osteoid islands (Fig. 2f) were found in the lamina propria of the lower part of mucosa.

Table 1. Anamnesis data and gross appearances of gastric polyps.

No.	Breed	Sex/Age (years)	Pathological Diagnoses/Cause of the death	Polypoid gastric masses	
				No. of GP/ Size (cm)	Gross appearance
1	Fox Terrier	M[a]/12	Cirrhosis/Chronic liver failure	3/0.8-1.5	Sessile, distinct border
2	German Shepherd	M/9	Euthanized/Chronic renal failure	7/0.3-0.5	Sessile, distinct border
3	Boxer	M/10	Hypovolemic shock/Traumatic hemorrhages	1/ 0.5	Sessile, no distinct border
4	Cocker Spaniel	F[b]/10	Pulmonary edema/Heart chronic failure	3/0.7-1.0	Sessile, no distinct border
5	Irish Setter	F/12	Euthanized/Metastasis mammary cancer	3/1.8-2.5	Sessile, no distinct border
6	Poodle	M/13	Cerebral infarction/Vegetative Endocarditis	1/0.8	Sessile, no distinct border
7	Mixed breed	F/16	Septicemia/Purulent endometritis	2/0.7-1.5	Sessile, distinct border
8	Argentinean mastiff	F/9	Cirrhosis/Chronic liver failure	1/3.2	Pedunculated
9	Rotweiller	F/12	Lumbar spondyloarthrosis/Euthanized	1/1.0	Sessile, no distinct border
10	Peckingese	M/13	Euthanized/Systemic mineralization/Hypothyroidis	1/0.4	Sessile, no distinct border
11	Poodle	F/16	Euthanized/Chronic renal failure/Renal fibrosis	9/0.8-1.5	Sessile, distinct border, basal notch
12	German Shepherd	M/15	Pulmonary edema/Heart chronic failure	1/1.2	Sessile, distinct border
13	Rotweiller	F/14	Euthanized/Metastasis osteosarcoma	1/2.0	Sessile, no distinct border
14	Mixed breed	M/17	Ethylene glycol intoxication	1/0.7	Sessile, no distinct border
15	Labrador	M/10	Restrictive pericarditis/Pulmonary edema	1/1.2	Sessile, distinct border

[a]M: male; [b]F: female

Table 2. Histological findings of gastric polyps and adjacent mucosa.

No.	Histological type of gastric polyps	Histologic special features	Adjacent gastric mucosa	H.spp[b] Score/Hp[c]
1	Hyperplastic	AB/PAS positive Goblet cells, low dysplasia	Moderate,active, superficial CG[a]	[d]3/Hp+
2	Inflammatory	Low dysplasia of foveolar epithelium, osteoid islands	Severe, active diffuse CG	3/Hp+
3	Hyperplastic	Hyperplasia of foveole, nematode larvae	Moderate, active superficial CG, nematode larvae	3
4	Hyperplastic	Hyperplasia, elongation and cystic dilation of glands, bone formation	Severe, active superficial CG	2
5	Hyperplastic	Hyperplasia, elongation and cystic dilation of glands	Severe, active diffuse CG	3
6	Inflammatory	Low dysplasia of foveolar epithelium;	Severe, active diffuse CG	2/ Hp+
7	Hyperplastic	Hyperplasia, elongation and branching of the foveolae	Severe, superficial CG	3
8	Hyperplastic	Low grade dysplasia of foveolar epithelium	Severe, active diffuse CG	3
9	Inflammatory	Low grade dysplasia of foveolar epithelium	Severe, active diffuse CG	3
10	Hyperplastic	AB/PAS positive Goblet cells	Severe, active diffuse CG	3
11	Hyperplastic	Hyperplasia, chronic inflammation AB/PAS positive Goblet cells	Severe,active superficial CG	3
12	Hyperplastic	Hyperplasia, elongation and branching of the foveolae	Severe, active diffuse CG	3
13	Hyperplastic	Hyperplasia, elongation and branching of the foveolae	Severe, active diffuse CG	1
14	Hyperplastic	Hyperplasia, elongation and branching of the foveolae	Severe,active superficial CG	2
15	Hyperplastic	Hyperplasia, elongation and branching of the foveolae	Severe, active diffuse CG	2

[a] CG: chronic gastritis; [b] H.spp: *Helicobacter* species; [c] Hp: *Helicobacter pylori*; [d] 1, 2, 3 represent intensity of colonization with H.spp; Hp+ (presence of *Helicobacter pylori*-like bacteria).

Pathological findings of canine gastric polyps

According to WHO classification (Head et al., 2003), three polyps (20%) were classified as inflammatory (benign lymphoid) polyps, based on the following findings: minimal elongated and branching hyperplastic foveolar epithelium, covering a well vascularized granulation tissue core (Fig. 3a). Severe inflammatory infiltrate, lymphangiectasia and edema of superficial lamina propria, marked fibrous tissue proliferation (Fig. 3b), glandular atrophy and severe cystic dilation were also observed. The abundant infiltrate consisted of mixed inflammatory cells, including lymphocytes, plasma cells, Mott cells (Fig. 3c), foamy macrophages (Fig. 3d), eosinophils, sparse distribution of neutrophils and multiple hyperplastic lymphoid follicles with prominent germinative centers (Fig. 3e). Mild epithelial dysplasia characterized by foci of multilayer epithelial cells, presenting hyperchromatic and large nuclei, sometimes with loss of polarity and multiple and prominent nucleoli, and scattered mitotic figures were also observed in the glandular and foveolar epithelium in all cases (Fig. 3f). In another two cases, low grade dysplasia of the foveolar epithelium and multiple, small and well circumscribed foci of dystrophic mineralization were identified in subepithelial location.

The adjacent gastric mucosa of both types of the GP had features of chronic gastritis (Fig. 4a) and a moderate to severe colonization by spiral rod bacteria, 3 to 6.0 μm in length, compatible with *Helicobacter* spp. (Fig. 4b). The gastric mucosa nearby the hyperplastic polyps showed severe chronic gastritis in 9 of 11 cases (81.8%) and moderate chronic gastritis in 2 of 11 cases (28.2%). The gastric mucosa surrounding inflammatory polyps presented severe chronic gastritis in all cases.

Giemsa stain revealed *Helicobacter* spp. in both types of GP and in the adjacent mucosa in all cases (Fig. 4c). A high density bacterial colonization was observed in 7 hyperplastic GP and in all the cases of inflammatory GP.

Immunohistochemistry using a polyclonal antibody identified the presence of *Helicobacter* spp. in all cases. Several brown marked organisms with small spiral-curved shape were identified and classified as *H. pylori* - like bacteria (Fig. 4d) in one hyperplastic GP and in two inflammatory GP (cases 2 and 6). *Helicobacter* spp. colonization was found only along the mucosal surface (grade 1) in 2 cases and also in the glandular lumen (grade 2) in 12 cases. No bacteria were identified inside the gastric epithelial cells. In a single case of hyperplastic GP, a nematode larve was present in the luminal portion of gastric glands (Fig. 4d).

In the adjacent gastric mucosa of all GP cases, Kendall test analysis showed no correlation between *Helicobacter* spp. colonization degree and gastritis scores (τ=0289; p=0.204). A significant correlation was found between *Helicobacter* spp. location and gastritis scores (τ=0.497; p=0.035).

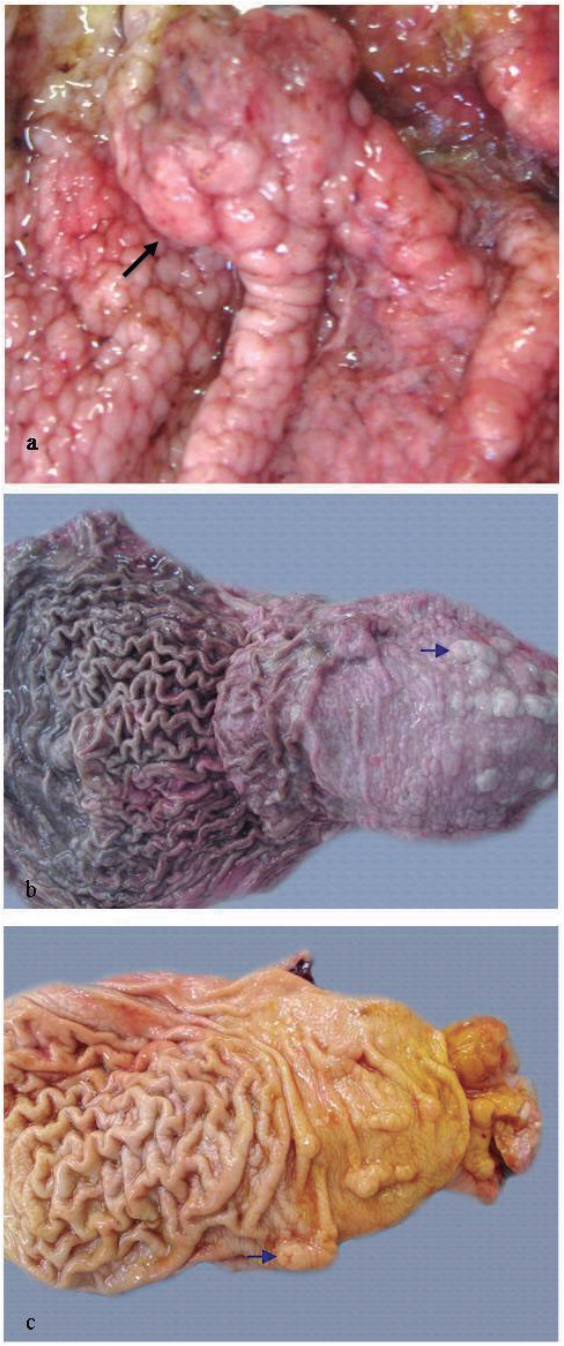


Fig. 1. Gross aspect of gastric polyps: A solitary hyperplastic polyp (arrow) associated with chronic gastritis in the pyloric region **(a);** Chronic and diffuse hypertrophic gastritis and multiple inflammatory polyps in the pyloric region (blue arrow) **(b);** Multiple hyperplastic polyps in the pyloric area and a polypoid mass with necrotic epithelial surface at corpus/pyloric junction (arrow) **(c).**

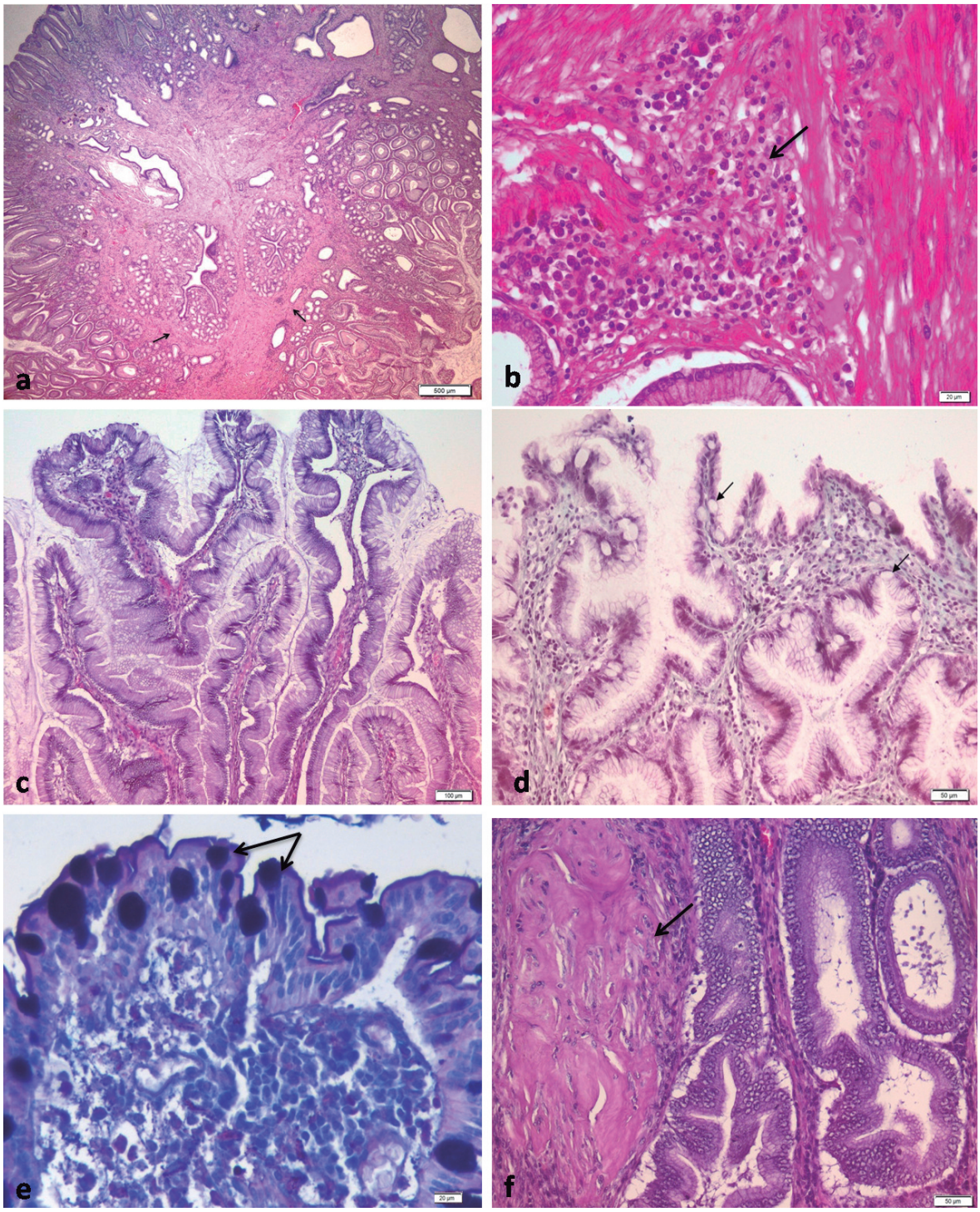


Fig. 2. Histopathological features of the hyperplastic gastric polyps: Severe hyperplasia of foveolar epithelium, cystic dilated glands and a marked core of fibrous and smooth muscle tissues (arrows) **(a),** with a low inflammatory infiltrate represented by mononuclear cells (arrow) **(b);** hyperplasia, elongation and branching of the non-neoplastic gastric foveolae **(c);** goblet cells hyperplasia resembling intestinal metaplasia (arrows) of foveolar epithelium **(d),** which was confirmed by Alcian blue/PAS combination staining and showing characteristic blue-purple mucin droplet of metaplastic intestinal goblet cells at antral foveolar epithelium level of stomach (black arrows) **(e);** the deep part of GP showed an island of heterotopic ossification with metaplastic osteoid and osteoblasts (arrow) **(f)**. (a, b, c and f: HE stain; d: MT stain; e: Alcian blue/PAS stain. Bar: a, 500 μm; c, 100 μm; d and f, 50 μm; b and e, 20 μm).

Pathological findings of canine gastric polyps

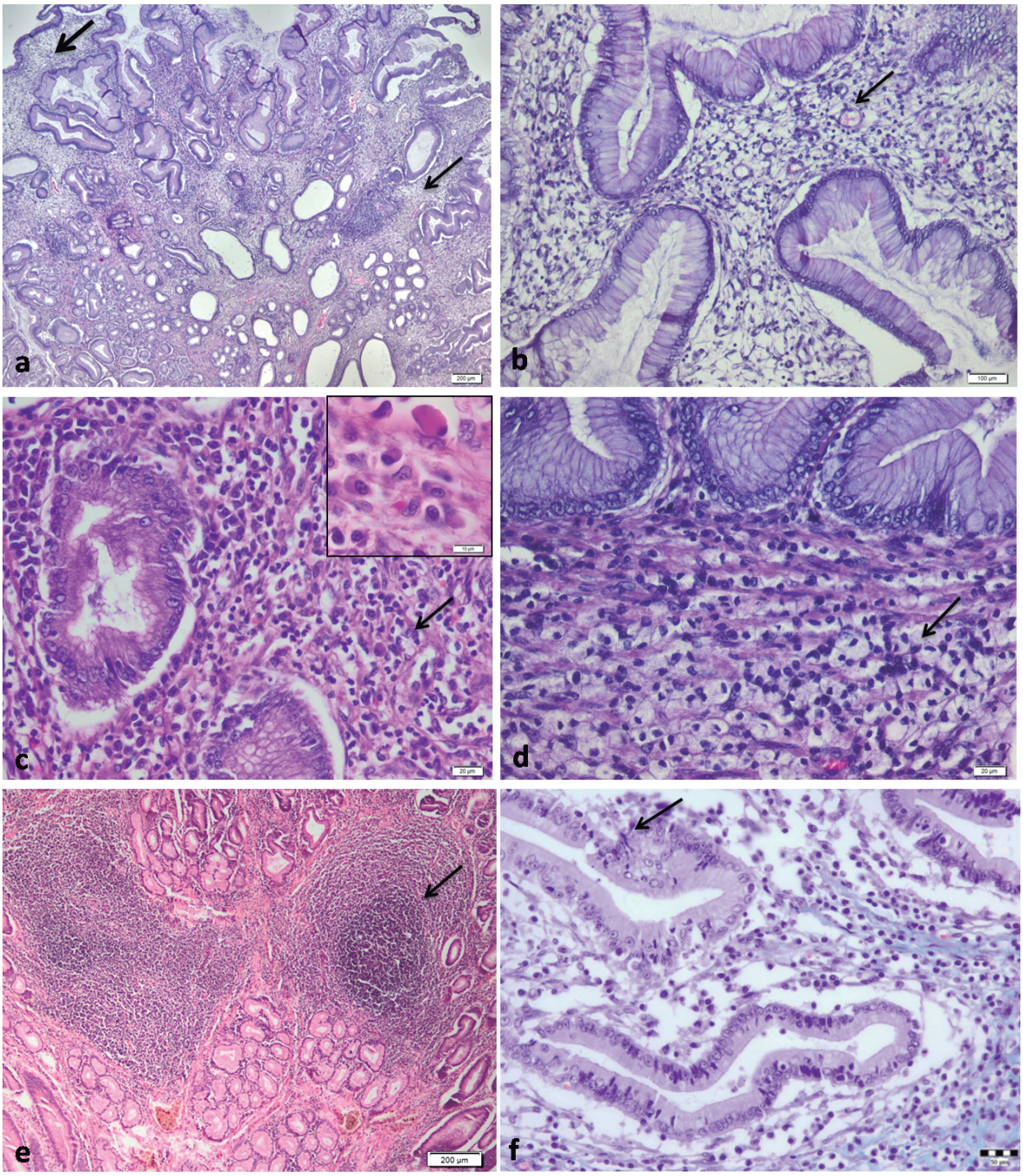


Fig. 3. Representative photomicrographs showing the histopathological features of the inflammatory gastric polyps: The polypoid growths showed a moderate foveolar hyperplasia covering a mesenchymal core (arrows) **(a)**, composed of a mixture of spindle cells, collagen fibers, small blood vessels (arrow) **(b)**, and severe infiltrated (arrow) with lymphocytes, plasma cells, Mott cells (the inset) **(c)** and several foamy macrophages (arrow) **(d);** lymphoid follicles proliferation with marked germinal center (arrow) **(e)** and low grade dysplasia of the foveolar epithelium (arrow) **(f)**. (a, b, c, d and e: HE stain; f: MT stain. Bar: a and e, 200 μm; b, 100 μm; c and d, 20μm f, 50μm; the inset (c), 10 μm).

Discussion

In the present study the prevalence of non-neoplastic gastric polyps was higher than that previously reported (Van der Gaag, 1988; Gualtieri et al., 1999).

Since GPs were identified by necropsy, the animal history concerning these gastric lesions remained unknown. No sex predisposition has been found in this study or in previous ones (Conroy, 1969; Diana et al., 2009; Kuan et al., 2009). Breed does not seem to affect the incidence of the GPs in our study since no French bulldogs, the only susceptible breed described in literature, were studied (Happe et al., 1977; Gualtieri et al., 1996).

Gastric polyps in these dogs were located in the pyloric area of the stomach, corroborating data previously reported (Happe et al., 1977; Diana et al., 2009; Kuan et al., 2009). The fundic and cardiac gland regions were not affected.

In contrast to human pathology, where several types of non-neoplastic gastric polyps, including hyperplastic, inflammatory fibroid, xanthoma, hamartomatous of the Peutz-Jeghers type, juvenile, gastric polyps in Cowden disease and gastric polyps in Cronkhite-Canada syndrome are described (Park and Lauwers, 2008), in domestic animals there is a lack of data about the histological features of gastric polyps. Based on microscopical findings, two gastric polyps have been

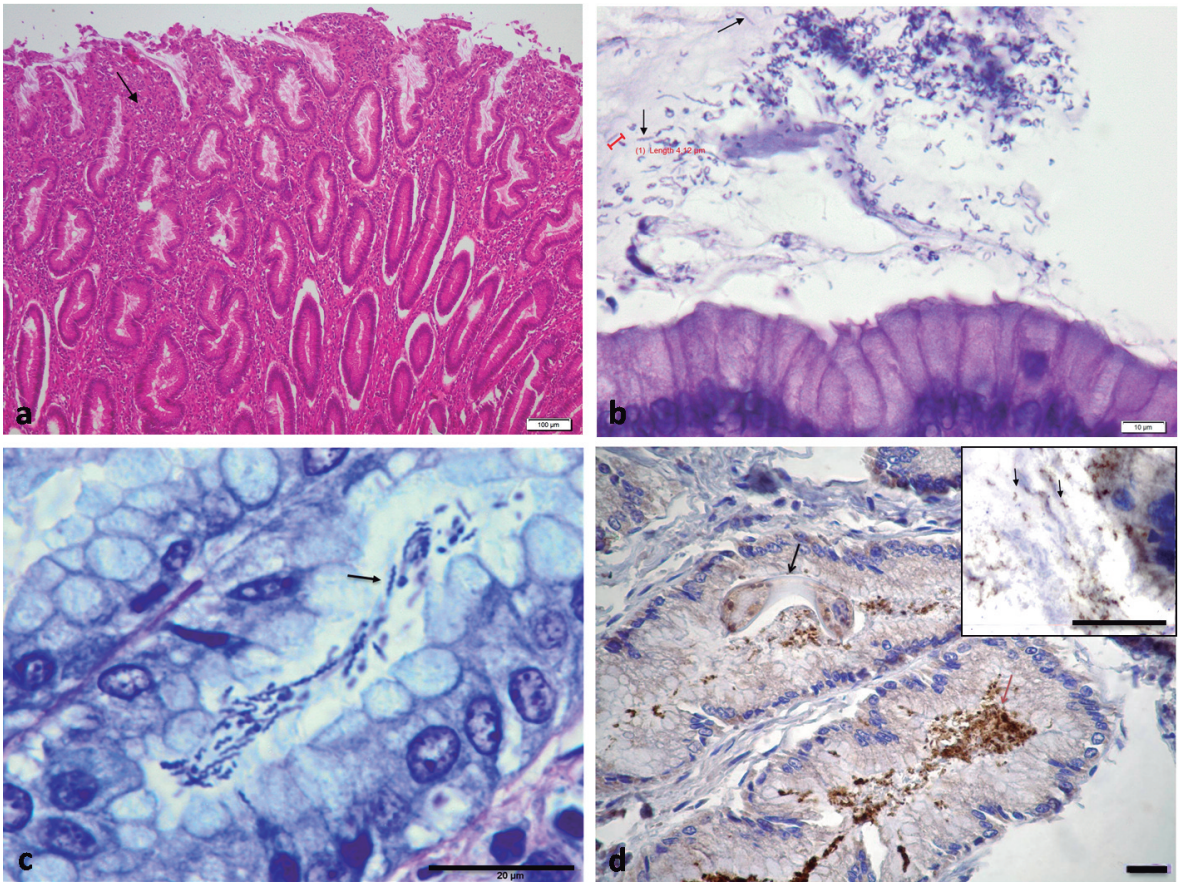


Fig. 4. Histopathological features of the adjacent gastric mucosa of the gastric polyps. The antral gastric mucosa showing a marked epithelial hyperplasia of the fovea and moderate to severe infiltration with mononuclear cells in the lamina propria (arrow) **(a);** several spiral-curved shaped bacteria (arrows) are present within the mucus layer on the epithelial surface of antral mucosa **(b);** several corkscrew-shaped bacteria, blue-purple stained, consistent with *Helicobacter* species (arrow) are present in foveolar lumen of the antral mucosa **(c);** immunohistochemistry based on polyclonal antibodies showing marked colonization of *H. pylori* brown stained on epithelial surface of the gastric mucosa (red arrow). The inset shows a suggestive morphology for *H. pylori* - like bacteria (arrows). A large and curved nematode larva (black arrow) is present in gastric foveole (**d**). a and b: HE stain; c: Giemsa stain; d: IHC, Haematoxylin counterstain. Bar: a, 100 μm; b, 10 μm; c, 20 μm; d and the inset, 50 μm.

distinguished: hyperplastic and inflammatory (benign lymphoid) (Head et al., 2003).

In the present study, GPs were classified according to WHO (Head et al., 2003) and histological features were the same as those described in previously reported cases (Gualtieri et al., 1996; Head et al., 2003; Kuan et al., 2009). Unlike the hyperplastic polyp, which is characterized by a branching core of lamina propria with some smooth muscle extending from the muscularis mucosa, covered by hyperplastic foveolar epithelium, sometimes with intestinal metaplasia, the inflammatory type is composed of normal epithelium covering a granulation tissue core infiltrated by several types of inflammatory cells. In other cases the epithelium covers foci of lymphocytes with well-differentiated germinal centers (Head et al., 2003). The most common type found was the hyperplastic polyp (80%) while the inflammatory type was found less frequently (20%). This agrees with the results previously reported in humans (Abraham et al., 2001) and other reports in dogs (Gualtieri et al., 1996; Diana et al., 2009). Malignant transformation of hyperplastic polyps and adenomatous lesions has been reported in both humans (Orlowska and Kupryjanczyk, 2002) and dogs (Conroy, 1969; Gaultieri et al., 1996). In this study, foci of preneoplastic lesions, such as multifocal areas of glandular atrophy and low to moderate epithelial dysplasia were found in both hyperplastic and inflammatory polyps. Foci of intestinal metaplasia were only identified in the hyperplastic lesions. In human pathology, the prevalence of true dysplasia arising in hyperplastic polyps is debated and reported rates have varied from 1.9% to 19% (Dirschmid et al., 2006).

In dogs, the etiology of GP is still unknown, but in humans an association between hyperplastic polyps and *H. pylori*-induced gastritis has been proposed (Wauters et al., 1990). Abraham et al. (2001) suggested that an exaggerated regenerative response to mucosal damage occurs, in the form of foveolar hyperplasia, leading to the development of a polypoid overgrowth. Additionally, *H. pylori* is responsible for gastric mucosa inflammation and it is associated with increased expression of interleukin 1-beta (IL-1ß), inducing chronic gastritis that plays an important role in the development of hyperplastic polyps by increasing epithelial cell turnover (Ji et al., 2006). Moreover, it seems that hyperplastic polyps regress after eradication of *H. pylori* in most human patients with *H. pylori* associated gastritis (Ohkusa et al., 1998).

The prevalence of *Helicobacter* spp. infection is high in dogs: it is seen in 61-80% of dogs presented to the clinician for the investigation of chronic vomiting and 67-100% of clinically healthy pet dogs (Eaton et al., 1996). Canine natural infection with *H. pylori* is disputed, although the PCR amplification method reveals the presence of these bacteria in the gastric mucosa of some dogs (Buczolitis et al., 2003; Ogawa et al., 2005).

On histological sections, there are two main groups of *Helicobacter* which can be distinguished based on morphologically aspects. The first group includes species similar to *H. pylori*, with few, loose helical turns and 2-4 μm in length, while the second group refers to larger spiral-shaped *Helicobacter* species, such as *H. felis* and *H. heilmannii-like* organisms, which are 7-10 mm long (Jakob et al., 1997). *In vivo*, *H. pylori* is an S-shaped bacterium with 1 to 3 turns, and 5x0.5 μm in length (Jones et al., 1985).

Compared with human pathology where the immunohistochemistry using polyclonal antibodies for *H. pylori* is considered a useful diagnostic tool, in veterinary medicine immunohistochemistry has been infrequently employed as a diagnostic assay (Jakob et al., 1997).

In this study both Giemsa and immunohistochemistry methods revealed *Helicobacter* spp. infection in all cases of GPs. A high degree of colonization was present in the majority of cases. Immunohistochemistry method using a polyclonal antibody was a very sensitive method, but was not able to discriminate among *Helicobacter* spp., all these species being stained. However, in three cases the morphology of the stained bacteria was suggestive of *H. pylori* - like organisms and was different from other non - *H. pylori* organisms.

The present observations indicate a high degree of antigenic homology among the different *Helicobacter* species in dogs. As with similar findings, the immunohistochemistry method using polyclonal and monoclonal antibodies anti *H. pylori* in pets can be used only for identification of gastric *Helicobacter* spp. infection (Scanziani et al., 2001).

In dogs with natural *Helicobacter* spp. infection, mild gastritis with infiltration of lymphocytes and plasma cell, and glandular degeneration has been commonly observed. Gnotobiotic dogs that have been experimentally infected with *H. pylori* and *H. felis* revealed mild gastric inflammation and lymphoid follicles (Radin et al., 1990).

The present study found that a severe gastritis in the adjacent mucosa is associated with GP development; the degree of inflammatory changes was not correlated with bacteria colonization density, but with the location of bacteria, suggesting that organisms that invade the glands produce more severe inflammation. These findings corroborate earlier reports that associate *Helicobacter* spp. infection with chronic gastritis in dogs (Hermanns et al., 1995). In contrast to our results several reports demonstrated the association between *Helicobacter* infection and chronic gastritis in dogs (Cattoli et al., 1999; Sapierzyński et al., 2003; Hernandez et al., 2007). Although, Lanzoni et al. (2011) revealed a differential distribution of *H. bizzozeronii* and *H. felis* in the fundic mucosa of Beagle dogs, and their intracellular localization within parietal cells and macrophages suggests a novel mechanism for the development of immune response and maintenance of chronic gastritis in dogs.

In conclusion, we report 15 cases of gastric benign

polyps in dogs located in the pyloric region of stomach. The most common GPs type was the hyperplastic, while inflammatory type was less common. All GPs occurred in dogs with severe or moderate antral chronic gastritis but unlike that described in human pathology, the pathogenetic links between these lesions are still controversial. The existence of epithelial metaplasia and dysplasia, in both hyperplastic and inflammatory polyps may suggest that these lesions present features of potential neoplastic transformation, although this hypothesis needs further studies and confirmation. Whether the *Helicobacter* species infection or other agents such as nematodes are directly involved in the development of chronic gastritis and GPs still remains an important question for further studies in this research field. Even so, the real pathogenic implications of the presence of *Helicobacter pylori*-like organism in canine gastric mucosa and its potential zoonotic risk need to be clarified.

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