UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

MEASUREMENT OF ANTI-BOVINE SERUM ALBUMIN ANTIBODIES IN DOGS WITH CHRONIC ENTEROPATHY

ANA CAROLINA SANTOS DE CASTRO

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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“Success is no accident. It is hard work, perseverance, learning, studying, sacrifice and most of all, love of what you are doing or learning to do.”

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SUBMITTED ABSTRACT TO INTERNATIONAL CONGRESS

Part of this study was integrated to the following abstract, submitted to 2019 Congress of the European College of Veterinary Internal Medicine - Companion Animals.

Anti-LPS, anti-flagelline and anti-bovine albumin seroreactivity in dogs with Inflammatory Bowel Disease

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Inflammatory bowel diseases (IBD) are a common cause of chronic gastrointestinal disease in dogs. Similarly to human IBD, canine IBD is thought to result from an abnormal immune response against microbiota in susceptible patients. In IBD patients, increased gut permeability and Toll-Like receptor dysregulation may promote mucosal inflammation into the gut by allowing systemic passage of microbial products. Human IBD patients exhibit increased reactivity to bacterial antigens evidenced by increased circulating levels of specific IgG and IgA. The aim of the study was to measure and compare serum antibodies levels against flagellin, lipopolysaccharide (LPS) and bovine serum albumin (BSA) in IBD and healthy dogs. Fourteen dogs with IBD were included in this prospective observational study. Diagnosis was based on clinical signs of at least 3 weeks' duration, the presence of lymphoplasmacytic and/or eosinophilic inflammation on intestinal biopsies and exclusion of other causes of chronic gastrointestinal signs. Eleven healthy dogs were included in the control group. The anti-flagelline, anti-LPS, anti-BSA IgG and IgA levels were measured by ELISA in the sera. A reference serum was included in each experiment to standardize the results.

Anti-LPS and anti-flagellin IgG levels were significantly higher in IBD dogs compared to control dogs ($p<0.05$ for both). Anti-BSA IgG levels were significantly lower in IBD dogs when compared to the control group ($p<0.05$). No differences were found for anti-flagellin IgA and anti-BSA IgA levels. The sensitivity of the anti-LPS IgA assay was found to be insufficient to allow an acceptable quantification of these antibodies.

Our study demonstrates elevated anti-bacterial antibody levels in IBD dogs and supports the current hypothesis that IBD in dogs is also characterized by an excessive immune response against the microbiota. These findings open the prospect to identify novel biomarkers of IBD in dogs.

DETECÇÃO E DOSEAMENTO DE ANTICORPOS SÉRICOS ANTI-ALBUMINA BOVINA EM CÃES COM ENTEROPATIA CRÓNICA

RESUMO

A Enteropatia crónica (EC) é uma doença complexa e multifatorial que se acredita ser resultado de uma resposta imunitária exacerbada a antigénios alimentares e/ou microbianos em animais geneticamente suscetíveis. A inflamação da mucosa intestinal é resultante de uma permeabilidade aumentada da barreira intestinal, o que permite a passagem de antigénios do lúmen para a circulação sistémica. Esta alteração culmina numa resposta imunitária exacerbada com consequente produção inapropriada de imunoglobulinas especificas. A proteína bovina é atualmente aceite como um dos principais desencadeadores imunitários em cães com EC.

O presente estudo visa comparar os valores de anticorpos específicos anti-BSA (IgA/IgG) em cães saudáveis e em cães diagnosticados com EC, estimando a potencial utilidade destes como biomarcador de alteração de permeabilidade intestinal ("leaky gut").

Foi efetuado um estudo do tipo caso-controlo que incluiu um total de 21 cães. Estes foram divididos em 2 grupos: grupo controlo (constituído por 8 cães adultos clinicamente saudáveis e alimentados com ração standard para adultos) e um grupo composto por 13 cães com sinais clínicos de EC. Em ambos os grupos foram medidos os anticorpos anti-BSA, através das amostras de soro obtidas e recorrendo a um kit ELISA espécie-específico.

Relativamente aos níveis séricos de IgA, não houve diferença significativa entre os dois grupos ($p=0,119$). No entanto, os níveis de IgG foram significativamente inferiores em cães com EC, quando comparados ao grupo de controlo ($p=0,046$).

O decréscimo observado em cães com EC pode ser explicado por uma eventual produção insuficiente de imunoglobulinas, consequente de uma fraca resposta humoral em relação aos antigénios bovinos. Outra possível explicação para o facto de os níveis de IgG serem mais elevados em cães saudáveis pode residir na origem da proteína animal da dieta, que poderá conter extratos bovinos.

Embora se defenda que a proteína bovina seja um dos maiores estímulos imunitários em cães com EC, estes resultados sustentam que os anticorpos anti-BSA não aparentam ser biomarcadores adequados a esta condição. Este trabalho corrobora estudos anteriores que questionam o uso de perfis séricos sorológicos para avaliar a potencial antigenicidade alimentar. São necessários mais estudos para esclarecer se baixos títulos de IgG poderão estar relacionados com uma deficiente resposta imune humoral em cães com CE.

Palavras-Chave: Enteropatia crónica, permeabilidade intestinal, imunoglobulinas, proteína bovina

MEASUREMENT OF ANTI-BOVINE SERUM ALBUMIN ANTIBODIES IN DOGS WITH INFLAMMATORY BOWEL DISEASE

ABSTRACT

Chronic Enteropathy (CE) is a complex disease that is thought to result from an overstated immune response against alimentary and microbiota antigens in susceptible animals. The amplified inflammation of the mucosa promoted by the increased and abnormal gut permeability in sick patients known as 'leaky gut', allows the antigens to pass through the systemic circulation, culminating in an unbalanced excessive immune response that is followed by the production of specific Immunoglobulins (Ig).

Bovine protein is historically believed to be one of the highest immune triggers in dogs with CE. However, studies about the use of anti-bovine serum albumin antibodies (anti-BSAA) as potential biomarkers of a disrupted immune-response in dogs with CE are lacking. This study aimed to compare anti-BSA antibody values (IgA/IgG) between healthy dogs and dogs diagnosed with CE, assessing its potential utility as a biomarker of a leaky gut.

A case-control study was conducted including a total of 21 dogs, divided into two distinct groups. The first group consisted on 8 client-owned healthy adult dogs (control group), fed with current adult standard dry diet; the second group was composed by 13 client-owned dogs with clinical signs of CE that historically contacted with standard diets of bovine-protein but were latter fed with an hydrolyzed bovine-free diet for at least two weeks. Serum samples were obtained and anti-BSA Antibodies (IgA/IgG) measured, using a species-specific ELISA kit.

There was no significant difference on IgA levels between groups (p= 0,119). Concerning IgG, serial titers were lower in diseased dogs, when compared to the control group (P=0.046).

Anti-BSAA do not seem to be good biomarkers of "leaky gut". A possible explanation for the fact that IgG levels are higher in healthy dogs relies on the diet protein source, which can contain bovine extracts. Assuming that diseased dogs had previously contacted with bovine-proteins, other possibility is that dogs with chronic enteropathy are not able to produce enough immune-globulins, showing a poor humoral immunity towards bovine antigens. Accordingly to previous studies, these results also disbelieves the use of serologic serum profiles to assess potential dietary antigenicity.

Albeit bovine protein is historically believed to be one of the highest immune triggers in dogs with CE, these preliminary results supports that anti-BSAA do not seem to be good biomarkers of a "leaky gut". Further studies are needed to evaluate if low-IgG levels can be related with a poor humoral immune response in dogs with CE.

Keywords: Chronic enteropathies, leaky gut, immunoglobulins, bovine protein

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ABREVIATIONS

ACVIM - American College of Veterinary Medicine

ANCAs - Anti-Neutrophil Cytoplasmatic Antibodies

Anti-BSAA – Anti-Bovine Serum Albumin Antibodies

APCs – Antigen-presenting cells

aPTT - Activated Partial Thromboplastin Time

ARD – Antibiotic-responsive diarrhea

ARE – Antibiotic-responsive enteropathy

ATIII - Anti-thrombin III

C – Constant Region

cANCA – Cytoplasmatic-antineutrophil cytoplasmic antibodies

CCECAI – Canine Chronic Enteropathy Clinical Activity Index

cCP - Calprotectin

CE – Chronic Enteropathy

CFU - Colony-forming Units

CIBDAI - Canine IBD activity index

CRI – Constant Rate Infusion

CRP - C-Reactive Protein

DCs – Dentritic Cells

DI – Dysbiosis Index

EE - Exudative enteropathy

FRE – Food-responsive enteropathy

FMT - Fecal Microbiota Transplantation

GALT - gut-associated lymphoid tissue

GI – Gastrointestinal

H – Heavy Chain

HSA - Human serum albumin

IBD – Inflammatory Bowel Disease

IgA – Immunoglobulin A

IgG – Immunoglobuline G

IgM – Immunoglobuline M

IIF - Indirect Immunofluorescence

IL - Interleucin

INF-γ – Interferon gamma

IRE - Immunosuppressant-responsive enteropathy

kDa - Kilodalton

L - Light Chains

LPE - Lymphoplasmacytic enteritis

LPS – Lipopolysaccharide

MALT - Mucosa-associated lymphoid tissue

MAMPs - Microbe Associated Molecular Patterns

MHC - Major histocompatibility complex

mRNA - Messenger ribonucleic acid

NF-AT - Nuclear factor of activated T-cells

NF-kB – Nuclear factor kappa B

NLRs - Nucleotide oligomerization domain-like receptors

NOD1 – Nucleotide-binding oligomerization domain 1

NOD2 – Nucleotide-binding oligomerization domain 2

NRE – Non-responsive Enteropathy

pANCA - Perinuclear-antineutrophil cytoplasmic antibodies

PAMPs - Pathogen-associated molecular patterns

PBMCs - Peripheral blood mononuclear cells

PCR – Polymerase Chain Reaction

PLE – Protein-losing Enteropathy

PRRs - Pattern recognition receptors

PTT - Prothrombin Time

rRNA - Ribosomal Ribonucleic Acid

SIBO - Small intestinal bacterial overgrowth

SRE – Steroid Responsive Enteropathy

SNPs – Single nucleotide polymorphisms

sRAGE - Soluble Receptor for Advanced Glycation End Products

TCR – T-cell Receptors

TGF-β – Transforming growth factor beta

TLI - Serum trypsin-like immunoreactivity

TLR's – Tool-Like Receptors

TMF - Fecal microbiota transplantation

TNF-α – Tumor necrosis factor alfa

TNF- β - Tumor necrosis factor beta

TPJ - Tight junction proteins

V – Variable Region

WSAVA - World Small Animal Veterinary Association

α1-PI - α1–proteinase inhibitor

SECTION 1 - DESCRIPTION OF THE ACTIVITES PERFORMED DURING THE TRAINEESHIP

The author of this dissertation has performed the final traineeship of the Veterinary Medicine Master Degree at Oniris - École Nationale Vétérinaire, Agroalimentaire et de l'Alimentation and at the Referral Centre Hospitalier Vétérinaire Frégis, under supervision of Dr. Juan Hernandez and co-supervision of Rodolfo Oliveira Leal, fulfilling a total of six months (from the 15th of September to the 28th of February). Thereafter, an extra-curricular externship of two months at the Internal Medicine Referral Service at the Veterinary Teaching Hospital of the University of Lisbon was also performed.

In order to develop this dissertation, clinical research skills were also developed in IECM (L'Unité d'Immuno-Endocrinologie Cellulaire et Moléculaire) of Oniris - École Nationale Vétérinaire, Agroalimentaire et de l'Alimentation by contacting with laboratory work and participating in internal medicine specialized investigations. The student engaged in all the laboratory activities that related with the aspects of the technical work of the upcoming research project and received practical training on how to correctly approach the laboratory procedures. During the remaining of the internship, several serum samples were provided and then adequately manipulated and analyzed, following numerous technical steps as coating plates, operating reagents, incubation and measuring absorbances on the spectrophotometer by means of serological specific techniques as *ELISA* for the final purpose of measuring specific immunoglobulins.

Besides all the work directly related with the laboratory research, these training periods in all of the referred internship placements allowed the student to embody the organization and functioning of the internal medicine service and critical care, to practice diagnostic approaches, main technical gestures and management of the hospitalized animals as well as imaging and endoscopy notions.

While in the traineeship, the student had the possibility to have an active role on technical gestures, consultations, discussion of clinical cases – approach, diagnosis methods, differential diagnosis, treatment, prognosis – and to assist imaging exams such as radiography, computed tomography and ultrasound achieving the overall notions of hospital and clinical procedures. In addition, the trainee was able to witness and participate on an ongoing research protocol concerning emerging therapeutical methods on chronic enteropathy involving fecal microbiota transplantation.

SECTION 2 – BIBLIOGRAPHY STUDY

1. CHRONIC ENTHEROPATHIES IN DOGS

1.1 INTRODUCTION and DEFINITION

Chronic Enteropathy (CE) in dogs is a chronic and relapsing gastrointestinal (GI) tract disease that is thought to result from a massive inflammation of the gastrointestinal tract, traduced by an overstated immune response to genetic, environmental, alimentary and microbiota antigens in susceptible animals.

We can state as CE, when the duration of clinical signs such as vomiting, diarrhea, nausea, borborygmus, hyporexia, abdominal pain, nausea and/or weight loss persist for longer than 3 weeks and after extra-digestive causes (i.e. chronic kidney disease, liver disease, pancreatitis, exocrine pancreatic insufficiency and hypoadrenocorticism), infectious or parasitic diseases have been ruled out.

According to their clinical characterization, and based on their response to treatment, CE's embodies food-responsive enteropathies (FRE), antibiotic-responsive enteropathies (ARE), immunosuppressant-responsive enteropathies (IRE) and non-responsive enteropathies (NRE) reflecting the refractory cases (Dandrieux, 2016). On these last ones, re-examinations should verify the possibility of concurrent diseases that may be affecting the previous imposed treatment.

The amplified inflammation of the mucosa, which is promoted by the increased and abnormal gut permeability in sick patients known as 'leaky gut', can result in loss of protein (Dossin & Lavoué, 2011). In these cases, CE is considered a protein-losing enteropathy (PLE) or exudative enteropathy (EE).

Since the majority of the dogs will positively respond with a simple diet-changing (Allenspach, Wieland, Gröne, & Gaschen, 2007), a sequential trial is followed before taking more invasive and severe approaches such as endoscopy. However, if at the time of diagnosis severe clinical forms of CE disease and/or hypoalbuminemia is observed - as the clinical consequences of low levels of albumin are associated with raised anesthetic risk - or there is high suspicion of neoplasia, a more invasive approach with endoscopy and mucosal sampling for histopathological characterization is justified (Gaschen, Allenspach, & Priestnall, 2019).

Nonetheless, the histological importance on defining the choice of treatment plan is currently in discussion since studies have reported that Canine IBD activity index and Canine Chronic Enteropathy Clinical Activity Index (CIBDAI/CCECAI) can clinical improve without concurrent significant changes in the severity of histopathological GI mucosal lesions (Allenspach et al., 2018, 2007; Schreiner, Gaschen, Gröne, Sauter, & Allenspach, 2008). This finding is not applied to FRE cases (Walker et al., 2013). In light of these assessments, and if a specific

treatment plan has apparently no effect on histopathological lesions in these dogs, the diagnostic value of histopathological sampling is questioned. It is suggested that histopathologic observations should be repeated after several months instead of weeks, for better identify the mucosal aspects evolution (Gaschen et al., 2019).

1.1.1 CLASSIFICATION OF CHRONIC ENTHEROPATHIES in DOGS

1.1.1.1 CE versus IBD – which fits the best?

There is currently a discussion about using the term Inflammatory Bowel Disease (IBD) instead of CE in dogs. This is due to the fact that there is a massive variation in the treatment and prognosis between IBD in humans and CE in dogs.

IBD in humans involves two disorders that are characterized by the inflammation of the intestinal wall: Crohn's disease (CD) and ulcerative colitis (UC) (Dandrieux, 2016). Although the etiology may be comparable, the biggest controversy about comparing CE in dogs and IBD in humans, comes from their response to the treatment. Previous studies reported that the highest percentage of the dogs positively responds to a diet-change (food-responsive therapy), smaller percentage responds to antibiotics treatment and a lower one improves with immunosuppressants (Jergens & Simpson, 2012). On the other hand, immunosuppressant therapy use is prioritized in human IBD to lower the inflammation and induce the healing of the intestinal mucosa (Grevenitis, Thomas, & Lodhia, 2015).

Additionally, in dogs with relapsing CE, surgery is hardly considered in contrast to human IBD that often requires resection surgery of the intestinal portions affected (Dandrieux, 2016; Hwang & Varma, 2008; Kilpinen et al., 2011; Vester-Andersen et al., 2014).

Therefore, CE is believed to be the term that should be used to characterize the disease in order to avoid misinterpretations. When utilizing CE, we are considering dogs with gastro-intestinal clinical signs with suspicion of intestinal inflammation but lacking of histological diagnosis. Also, using this term does not infer which treatment will be implied (Dandrieux, 2016).

According to this, we reserve the term IBD for dogs where intestinal and extra-intestinal causes of chronic inflammation have been excluded, refractive to diet changing and antibiotic trials and with confirmed cell infiltrate of the intestinal mucosa (i.e. endoscopic or laparoscopic biopsies) and immunosuppressants drugs should be prescribed while the etiology is still unknown (Dandrieux, 2016). Therefore, the term IBD should be used for idiopathic chronic enteropathy, as IBD can only be diagnosed in dogs with histologic evidence of inflammation and all other disorders with known etiological causes were already excluded, meaning it can be considered idiopathic (Allenspach et al., 2007; Dandrieux, 2016; Simpson & Jergens, 2011; Walker et al., 2013; Willard, 2016).

1.1.1.2 Characterization of CE's

a) Food-Responsive Enteropathy

Food-responsive enteropathy consists on the CE in which the animals respond to diet-changing therapy alone. FRE it is confirmed to be where the majority of the CE dogs belong, with an average of 66% of the cases (Allenspach, Culverwell, & Chan, 2016; Allenspach et al., 2007; Mandigers, Biourge, Van Den Ingh, Ankringa, & German, 2010; Marks, Laflamme, & McAloose, 2002; Volkmann et al., 2017).

FRE (as well as ARE) is more likely to occur in younger animals in comparison to the others CE's forms as Steroid-responsive enteropathy (SRE) - a subgroup of IRE, that characterizes dogs that respond to prednisolone (Dandrieux, 2016) -, being more frequent at 3 years (Allenspach et.al, 2007, 2016). Additionally, FRE dogs have usually lower CCECAI scoring and normal levels of serum albumin when comparing to ARE and SRE (Allenspach et al., 2007; Dandrieux, 2016) and often present large-bowel clinical signs with present hematochezia, tenesmus, increased frequency of defecation mucus and decreased fecal volume (Allenspach et al., 2007). Usually, FRE is also associated with low clinical disease activity, meaning lower clinical scores reflecting less severe forms of the disease.

Concerning this group, diseased dogs will have a significant and quickly – an average of two weeks - clinical response after initiating a new hydrolyzed protein diet or a commercial or home-cooked novel protein source. If an appropriate and stable clinical response under elimination diet trial is verified during the follow-ups, a return to their original diet may be attempted as Allenspach et al. (2007) reports that 31 of 39 dogs that were switched back to their original diet did not develop clinical signs of disease for up to 3 years after diagnosis. However, if there is a clinical relapse, the strict elimination diet should be continued.

b) Antibiotic-Responsive Enteropathy

ARE was believed to historically be associate to small intestinal bacterial overgrowth (SIBO), which was defined as a syndrome of chronic diarrhea decurrent from an unbalanced proliferation of the intestinal microbiome that positively responds to the administration of antibiotics. Currently, the initial reports of an increased number of bacteria have been recognized as fallacious, reflecting that maybe there is no real overgrowth (Suchodolski, 2011), reason why the name ARD is currently preferred (German, Day, et al., 2003; Hall, 2011). Although in humans the limit for normal duodenal bacteria numbers is stated at ≤ $1x\ 10^5$ cfu/mL (colony-forming units (CFU) per milliliter (ml)) of duodenal juice, there is still disagreement on the extrapolated value for dogs since a much higher counts had been observed in clinically healthy dogs (Hall, 2011; Karen, 1999; Willard et al., 1994). SIBO is now recognized as one of the consequences of a chronic inflammation of the intestine, that is also often associated to dogs with PLE (Hall & Day, 2017; Hall, 2011).

ARE often affects young animals from large dog breeds, among which German Shepherds are overly represented (Allenspach et al., 2016; German, Hall, & Day, 2003; Hall, 2011) as well as Sharpeis and Boxers (Allenspach et al., 2016). Clinical signs may vary or be a combination between small and large bowel signs, varying from small intestinal signs as polyphagia or noticeable anorexia, vomiting, intermittent large-volume diarrhea with or without weight loss to tenesmus, hematochezia and increased frequency of defecation (Suchodolski & Allenspach, 2019). Also, flatulence and borborygmus may reflect an excessive production of intestinal gas (Hall & Day, 2017). As bacteria can synthesize folate and compete and bind to available cobalamin, hypocobalaminaemia with concurrent serum folate concentration should be expected. However, studies have already reported the limited value of these parameters as no clear correlation was found between its values and ARE dogs (German, Day, et al., 2003). In ARE, relapses with chronic diarrhea are common and in higher frequency when compared to FRE and IRE groups, reflecting the need for repeating treatment and the difficulty related in achieving long-term remission (Allenspach et al., 2016).

The disrupted barrier with concurrent altered permeability of the gut allows the invasion of the intestinal mucosa by commensal agents and invasive pathogens. ARE usually respond to metronidazole, oxytetracycline or tylosin in short-term, whereas long-term efficacy has still few available data (Allenspach et al., 2016; Kilpinen et al., 2011). The usage of antibiotics plays an interesting role in regulating the intestinal flora and modulating the immune-response but the optimal duration of treatment is still under discussion (Dandrieux, 2016; Hall, 2011).

Although genetic factors such as mutations in pattern recognition genes (TLR4 and TLR5) may suggested genetic predisposition in some dogs with ARE, most articles refer to this state as idiopathic (Kathrani et al., 2012, 2010).

Granulomatous Colitis or Boxer's Histiocytic Ulcerative Colitis is the only affection that has been proved to have bacterial etiology, usually *Escherichia Coli* and full clinical resolution when applying enrofloxacin treatment. However, cases of resistance have been reported (Craven et al., 2011). It is usually diagnosed in young dogs (average of 4 years old) reflected by a massive inflammation of the intestine, and associated to a periodic acid-Schiff (PAS) positive macrophages and the existence of clusters of mucosal invasive *Escherichia coli* strains in Fluoresce in situ hybridization (FISH) analysis (Manchester et al., 2013; Simpson et al., 2006).

c) Immunosuppressant-Responsive Enteropathy

If there is no improvement of the clinical signs after the diet and antibiotic trial, immunosuppressant drugs should be introduced. IRE affects mostly middle-age to older dogs (Allenspach et.al, 2007, 2016). Some predisposal reported breeds are German Shepherds or Sharpeis (Hall & Day, 2017).

Although the most frequently observed clinical signs are chronic vomiting and diarrhea, episodes of anorexia or even polyphagia can also be verified, as well as weight loss, abdominal pain, flatulence, polyuria/polydipsia, presence of mucus on the stools, hematochezia, increased frequency of defecation, tenesmus and lethargy. SRE is associated with higher clinical indexes, revealing severe clinical forms of CE (Allenspach et al., 2007), sometimes subnormal albumin and cobalamin concentrations, endoscopic mucosal changes with variable degrees of histologic cellular infiltration within the biopsy samples. The diagnosis of SRE is sometimes challenging as it implies exclusion of other gastro-intestinal or extra-gastrointestinal disorders that can cause chronic GI clinical signs (Jergens & Simpson, 2012; Kent, 2017).

Handling of immunosuppressants before the obtention of samples for histopathological evaluation has historically believed to seriously compromise the possibility of a correct diagnosis (Hall & Day, 2017). After diet and antibiotic trial and always before prescribing immunosuppressants, more invasive techniques such as endoscopy or laparoscopy/laparotomy and sampling should be performed in order to allow the evaluation of the intestinal mucosal inflammation and a correct characterization of the cell infiltrate, as well as excluding neoplastic hypothesis such as lymphoma (Allenspach et al., 2016; Dandrieux, 2016; Jergens & Simpson, 2012; Washabau et al., 2010). Nonetheless, the usage of immunosuppressants and its influence on histopathologic findings is nowadays in discussion (Gaschen et al., 2019).

The treatment of SRE usually involves a combined approach between diet-changing and immunosuppressants. Usually the immunosuppressant drugs employed are prednisolone, azathioprine, budesonide or cyclosporin. They can be used alone or in combination. The benefit of the simultaneous utilization of antibiotics is still on discussion since the exclusive utilization of prednisone has shown no significant difference on efficacy when compared to the combination of this pharmaceutical drug with metronidazole (Jergens et al., 2010).

d) Protein-Loss Enteropathy

PLE is a complex syndrome that is consequence of the increased permeability of the intestinal mucosal, resulting in the loss of albumin and consequently decreasing oncotic pressure. This loss is faster than its synthesis in the liver, resulting in hypoalbuminemia. Other than CE, PLE can also be often related with intestinal lymphoma and primary or secondary intestinal lymphangiectasia or less likely with crypt lesions, obstructions, infectious enteritis (histoplasmosis) and gastrointestinal ulcers or erosions (Allenspach et al., 2007; Craven, Simpson, Ridyard, & Chandler, 2004; Dandrieux, 2016; Dossin & Lavoué, 2011; Peterson & Willard, 2003).

Usually the clinical presentation consists on chronic small-bowel diarrhea, sometimes vomiting and, according to the severity, weight loss, muscle dystrophy and edematous signs (Dossin &

Lavoué, 2011). However the clinical presentation can be extremely variable, and some animals can even have an apparent good clinical condition without any signs of CE (Willard, 2015).

Intestinal lymphangiectasia is characterized by dilation of the lymphatic vessels within the gastrointestinal tract, with leakage of lymph and its components - proteins, lipids and lymphocytes - into the intestinal lumen, Yorkshire Terrier, Sharpeis, German Shepherd, Maltese Terriers, Norwegian Lundehunds and Rotweillers are predisposal breeds to develop this condition. Lymphangiectasia can be primary (congenital or idiopathic) or secondary (acquired), being a consequence of other conditions (i.e. hypertension, inflammatory mucosal infiltration, intestinal neoplasia) that causes unbalanced oncotic pressure and lymph overflow, consequently causing severe and chronic protein-losing enteropathy in dogs (Davitkov et al., 2017; Dossin & Lavoué, 2011; Larson, Ginn, Bell, Davis, & Foy, 2012; Peterson & Willard, 2003). Additionally, a study reported a distinct familial predisposition for protein-losing enteropathy associated with a protein-losing nephropathy in 50% in Soft Coated Wheaten Terrier breed dogs. In addition, sensibility to alimentary allergens or immune-mediated diseases were also reported. Even with treatment, prognosis is usually poor (Littman, Dambach, Vaden, & Giger, 2000).

Orienting the diagnosis of PLE secondary to CE, proceeds by identifying the origin of the hypoalbuminemia. These dogs often reflect panhypoproteinaemia – hypoalbuminemia and hypoglobulinaemia – which predisposes for ascites, edema and a pro thrombotic state (Bota et al., 2016; Dossin & Lavoué, 2011; Gaschen, Marks, Zoran, & Williams, 2013; M. Willard, 2015), but isolated albumin loss can also be observed (Allenspach et al., 2007; Willard et al., 2000). Ionized hypocalcemia, high levels of PTH, hypovitaminosis D, hypocholesterolemia and increased fecal and lower serum α1–proteinase inhibitor (α1-PI), are also common findings (Gow et al., 2011; Willard, 2015).

A renal protein-loss and hepatic dysfunction should be excluded by performing a urinalysis and a bile acids stimulation test, respectively (Bota et al., 2016; M. Willard, 2015), ionized hypocalcemia - reported as secondary with the intestinal lipidic and vitamin D malabsorption (Allenspach, Rizzo, Jergens, & Chang, 2017) - and hypocholesterolemia occur in PLE secondary to CE as consequence of malabsorption, when in PLE secondary to renal protein-loss, an hypercholesterolemia is currently identified (Willard, 2015).

The prothrombotic state is a consequence of anti-thrombin III (ATIII) intestinal lost, and a platelet activation consequence of hypoalbuminemia and hyperfibrinogenemia. As so, specially in severe cases, the evaluation of the coagulation parameters (fibrinogen, anti-thrombin III (ATIII), Prothrombin Time (PTT), Activated Partial Thromboplastin Time (APTT), and D-dimers) or thromboelastography are also important although PLE dogs continue in an hypercoagulable state and as so, predisposed to thromboembolic complications despite a

good clinical improvement and response to treatment (Goodwin, Goggs, Chan, & Allenspach, 2010).

Serum α1–Proteinase Inhibitor (α1-PI) is a major proteinase inhibitor that is synthetized in the liver and it is reduced in mild to severe cases as a consequence of the increased permeability and protein-loss across the intestine. While serum α1-PI is founded to be decreased, fecal α1-PI is usually reported to be increased (Dossin & Lavoué, 2011; Murphy et al., 2003; Willard, 2015).

PLE diagnosis still relies, as the other CE, on histopathological confirmation (Dossin & Lavoué, 2011; M. Willard, 2015). Dogs with protein loss are confirmed to have a more reserved prognosis in comparison to the ones that have higher concentrations of serum albumin in other CE forms (Allenspach et al., 2007; Craven et al., 2004; Dandrieux, 2016).

Successful management of PLE implies treatment of the primary disease, nutritional and if needed, oncotic support as well handling of the secondary complications (Hill, 2013).

1.2 ETIOLOGICAL FACTORS

The etiology and pathogenesis of canine CE is complex and still uncertain. Currently, CE is believed to be consequence of a dysregulated host immune response triggered by environmental, microbiome, and dietetical originated antigens reacting on an abnormal mucosal barrier reflecting an immune-mediated origin (de Souza & Fiocchi, 2016; Kent, 2017). This can also be related to genetically-predisposed individuals that are suspected to have the integrity of the intestinal mucosal barrier compromised. The immunopathogenesis of CE will be addressed in detail later on this study.

1.2.1 Genetic and immunity factors - Breed Predisposition

Certain genetic deficiencies or variations result in inadequate innate and immune responses, failing on the recognition of commensal microbiota or on having an adequate signing and elimination of the invasive pathogens. Several studies have been developed concerning the genetic abnormalities and disarrangements in the pattern recognition receptors (PRRs) as they may be a factor on the triggering and perpetuation of intestinal inflammation. Toll-like receptors (TLRs) are pattern-recognition receptors that by recognizing the microbe associated molecular patterns (MAMPs) identify the physiological intestinal microbiota, maintaining the gut flora homeostasis (McMahon et al., 2010; Thaiss, Zmora, Levy, & Elinav, 2016). Mutations in this TLRs can compromise the homeostasis by not recognizing the commensal bacteria and distinguishing them as pathogenic antigens, consequentially losing tolerance and triggering an immune-response when these antigens start on crossing the intestinal mucosal barrier enhancing the inflammation (Allenspach et al., 2007; Jergens & Simpson, 2012).

In the paragraphs ahead, it will be presented specific forms of CE syndromes that are known to be associated with hereditary and genetic anomalies in specific breeds. German Shepherd, Basenjis, Border Collie, Weimaraner, Boxer, Rottweiler and Gordon Setters breeds are some of the breeds to have genetical predisposition to develop this conditions (Craven et al., 2011; Donnini, Rothschild, Walugembe, Jergens, & Allenspach, 2019; Jergens & Simpson, 2012; Kathrani et al., 2011).

Examples are:

- Histiocytic Ulcerative Colitis in Boxers – it is related to a NCF2 gene polymorphism. This gene is implied on the activation of NADPH oxidase, a crucial enzyme for the innate immunity on the elimination of intracellular pathogens (Craven et al., 2011). Recent studies identified in Boxer and French Bulldogs, identified a region in chromosome 38 encrypting genes responsible for the signaling of lymphocyte activation molecule family, involved in the sensing and killing of *E.coli* (Hayward et al., 2016);
- German Sheperd's - reported to have lower mucosal IgA concentrations, and nucleotide oligomerization domain 2 (NOD2) and TLR5 polymorphisms. A single nucleotide polymorphism (SNP) in NOD2 is also reported to be implied on the etiology of humans Crohn's and to be related to the innate immunity response (Kathrani et al., 2014). In this breed, significant upregulation of TL4 mRNA (messenger ribonucleic acid) expression and a downregulation of TL5 is consistently observed in dogs with the disease (Allenspach et al., 2010). Recently the same author reported that IL-13 and IL-33 mRNA in the duodenal mucosa are significantly under expressed in German Shepherd with IBD in comparison to other breeds with IBD and to healthy dogs, indicating that Th2 cytokines may be associated to the pathogenesis of IBD in these breed (Kathrani et al., 2019);
- Immunoproliferative Enteropathy in Basenjis, related to an inherited autosomal disease (Tizard, 2013);
- Protein-losing enteropathy/Protein-losing nephropathy Complex in Soft-coated Wheaten Terriers – also an inherited autosomal disease associated to a common male ancestor, where protein-losing enteropathy is associated in half of the cases to a protein-losing nephropathy, more often in middle-aged females but already described also in males (Littman et al., 2000);
- Diarrheal syndrome in Norwegian Lundehunds (German, Hall, et al., 2003);
- PLE in Gordon Setters, related to several SNP in genes belonging to chromosomes 10,12,15,17,18,21 and 23 (Donnini et al., 2019).

Additionally, Yorkshire and Rotweiller breeds are also often associated with PLE as a consequence of CE or due to primary lymphangiectasia.

There are also specific breeds with CE that are predisposed for allergenic intolerances such as gluten-sensitive enteropathy in the Irish Setters dogs related to a autosomal recessive inheritance pattern (Simpson & Jergens, 2011) and for nutrient deficiencies such as low cobalamin levels in Sharpeis.

These breed predispositions suggest an interaction between genetic factors and the host immunity response and enhance the relevance of the genetical influence on the development of chronic enteropathies.

1.2.2. Dysbiosis influence

A multiplicity of microorganisms – bacteria, fungi, viruses and other microbial and eukaryotic species - colonizes the intestinal mucosa, composing the intestinal microbiota and holding symbiotic relationships with the host, providing help in making the energy derived from the nutrients bioavailable and playing an important role on the immune defense by competing with invasive pathogenic bacteria and maintenance of tolerance to harmless antigens (Belkaid & Hand, 2014; Suchodolski, 2016; Tizard & Jones, 2018). Also, the microbiome has immune-modulating and protective functions with the host, enhancing the mucosal barrier and providing metabolites necessary for the host's metabolism. It also influences some morphological and functional features, by affecting the villus size, the enterocyte turnover and the motility of the small intestine. The disturbance of this homeostasis (e.g. sudden diet changes, decreased state of immunity, concomitant disease) may predispose the host to CE by the alteration of the population composition.

The role of enteric commensal and invasive bacteria and its impact on CE pathogenesis has been a target of several studies. As referred, there are evidences that an abnormal immune-response to the commensal bacteria is one of the factors that influence the development of CE in genetic susceptible animals (Rioux, Madsen, & Fedorak, 2005; Xenoulis et al., 2008), which is related to a lack of recognition of this bacteria as commensal and an ineffective removal of invasive bacteria as earlier described.

Dysbiosis is a concept that characterizes the imbalance between pro-inflammatory bacteria and anti-inflammatory bacteria. This dysbiosis is associated with alterations in the immunomodulatory bacterial metabolites (i.e. short chain fatty acids, secondary bile acids and tryptophan products) and of tolerance mechanisms. Bacteria exert potent immune-modulation signals as some are stimulators of Treg and Th17 cells or influence innate lymphoid and dendritic cells. Additionally, bacterial metabolites decurrent from bile acid and tryptophan metabolism such as indol also exercise their anti-inflammatory activity, reinforcing the mucosal barrier as well as the end product of fermentation of complex carbohydrates, short chain fatty acids, work as major source of energy to the host and as an inducer of enterocyte renewal and proliferation (Arpaia et al., 2013).

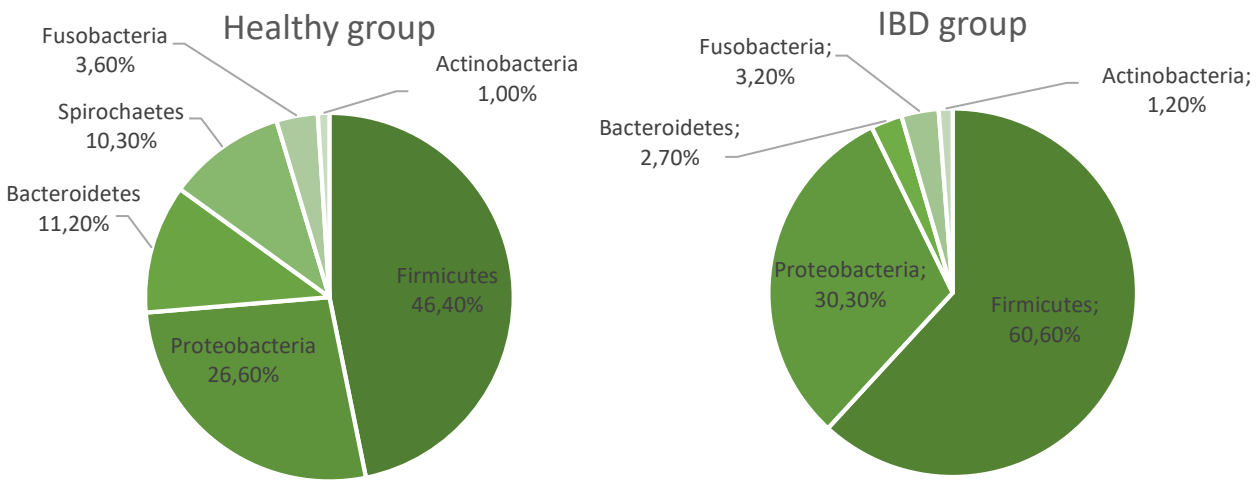
Commensals are known to be active inductors of regulatory immune-responses by actively suppressing the inflammatory responses to food or other originated antigens. (Belkaid & Hand, 2014; Suchodolski, 2016; Weiner, da Cunha, Quintana, & Wu, 2011). From these facts one may conclude that the lack of these bacteria leads to signaling processes that will cause alterations in the immune function (Arpaia et al., 2013; Duboc et al., 2013; Golubeva et al., 2017; Tizard & Jones, 2018).

Variances in the composition whether diversity or populations percentages - of the gut microbiota have been registered in human IBD patients, when compared to the healthy individuals (Barko, McMichael, Swanson, & Williams, 2018; Damman, Miller, Surawicz, & Zisman, 2012). The development of modern molecular analysis methods allowed to determine the composition of the microbiota, and consequently to verify in dogs with CE a dysregulation of the intestinal microbiota with alteration of the commensal populations. Whether these alterations are a cause of CE, a consequence of inflammation or both is still in discussion (Suchodolski, 2016; Xenoulis et al., 2008); however it is known to be an enhancement factor of inflammation. All in all, it is well known that although the microbiome has several similarities between the different individuals (confirmed by metagenomic analysis), each animal holds an exclusive microbial profile influenced by genetical and environmental factors (Guard & Suchodolski, 2016).

Several studies aimed to describe the changes on the microbial community of the affected dogs in comparison to healthy dogs using PCR (Polymerase Chain Reaction) amplification, Next-generation sequencing (still not available for clinical use) and Fluoresce in situ hybridization (FISH), but there is still no gold standard for evaluating the exact pattern of dysbiosis.

A study by Xenoulis in 2008 – figure 1 - aimed to compare the small intestinal microbial communities of healthy and IBD dogs reporting the alterations of the different microbiomes, using comparative 16S ribosomal ribonucleic acid (rRNA) gene analysis. This study revealed a decrease in the microbiome diversity (Xenoulis et al., 2008). Although the phylum *Firmicutes* (e.g., *Faecalibacterium*, *Ruminococcaceae*, *Turicibacter*, *Blautia*) and *Proteobacteria* were dominant in both groups, the percentage within each phylum increased in IBD group. A massive increase of the *Enterobacteriaceae* family, posteriorly identified as *E. coli* strains (phylum *Proteobacteria*) was observed in the affected dogs, which can predispose or influence for some forms of CE (e.g. ARE, Boxer's Histiocytic Ulcerative Colitis) (Manchester et al., 2013; Simpson et al., 2006; Xenoulis et al., 2008). This finding concerning E.coli goes in line with what previous studies had already stated concerning the microbiome characterization in both human and animal IBD (Darfeuille-Michaud et al., 2004; Frank et al., 2007; Rhodes, 2007; Schuppler, Lötzsch, Waidmann, & Autenrieth, 2004). Concerning *Bacteroidetes*, a decrement was observed - these bacteria are known to be beneficial for a normal function of the distal small intestine and their diminishment may worsen the clinical signs. Also, there was a severe decrease or even absence of specific families in comparison with healthy dogs. Additionally, a study stated in German shepherds affected with ARE, an increased population of *Lactobacillus* in comparison to the healthy dogs (Allenspach et al., 2010).

Figure 1 - *Characterization of the intestinal microbiome in healthy and IBD groups. Adapted from Xenoulis et al. 2008*



However, recent studies also using 16S rRNA gene sequencing come to disagree with Xenoulis (2008) by assessing a decrease in members of *Firmicutes* (i.e. *Faecalibacterium*, *Ruminococcaceae*, *Turicibacter*, *Blautia*), *Bacteroidetes* and *Fusobacteria* phylum's and an increase *in Proteobacteria (e.g. E. coli)* and *Actinobacteria* in CE dogs. Also, duodenal dysbiosis was correlated with the severity of histopathological scores. (Honneffer, 2014; Suchodolski, Dowd, Wilke, Steiner, & Jergens, 2012).

As so, canine dysbiosis has been demonstrated in canine IBD (Minamoto et al., 2015). The canine microbiota dysbiosis index (DI) is a recently developed rapid PCR-based assay that assesses microbial changes in fecal samples by quantifying the loads of 8 bacterial groups that are usually associated to CE dogs – Total bacteria*, Faecalibacterium, Turicibacter, Blautia, Fusobacterium, C. hiranonis Streptococcus* and *Escherichia coli*. This index allows to define a reference interval by rearranging the results in one single number. A DI< 0 indicates normobiosis, while a DI≥0 indicates dysbiosis (AlShawaqfeh et al., 2017; Suchodolski, 2017). According to AlShawaqfeh. the overall total bacteria, *Faecalibacterium, Turicibacter* spp*., Blautia* spp*, Fusobacterium* spp*, and C. hiranonis* were significantly decreased in the CE diseased group, while *E. coli* and *Streptococcus* were significantly increased. These findings confirm what previous studies have stated: *Faecalibacterium* spp and *Turicibacter* spp is decreased in dogs with IBD (Minamoto et al., 2015; Rossi et al., 2014; Suchodolski, Markel, et al., 2012; Vázquez-Baeza, Hyde, Suchodolski, & Knight, 2016), *Fusobacterium* is decreased in canine IBD, although it is reported to be high in human IBD (Alshawaqfeh, Bashaireh, Serpedin, & Suchodolski, 2017; Minamoto, Dhanani, Markel, Steiner, & Suchodolski, 2014; Vázquez-Baeza et al., 2016), *Blautia* is also significantly lower in IBD dogs while E. *coli* and S*treptococcus* were reported as increased.

Alshawaqfeh assessed in his essay that DI is not influenced by age, gender or bodyweight and that concerning the diet composition, the protein, fiber and fat content had no impact on the index attained. However, the author reported to have only dietary history of half of the dogs included in his study (AlShawaqfeh et al., 2017). Nonetheless, previous study had already stated that there is no correlation between the protein content and the dysbiosis index (Minamoto et al., 2015; Vázquez-Baeza et al., 2016). DI is also correlated with the clinical score, according to Canine Chronic Enteropathy Clinical Activity Index (CEECAI) (AlShawaqfeh et al., 2017). The particularities of this scoring system will further be approach.

According to Suchodolski, this DI value is not correlated to the type of CE and, as so, it does not permit to distinguish between FRE, ARE or IRE (Suchodolski, 2017). However, it is important to mention that 15% of the healthy dogs, as well as dogs on antibiotic therapy or with exocrine pancreatic insufficiency can also have an increased DI. Those 15% of the reported healthy dogs with increased DI are usually between 0-2 (Suchodolski, 2016). The clinical utility of DI to assess the dysbiosis in CE dogs and the interest in following the microbiotical changes overtime and in response to treatment (expecting DI<0, meaning normobiosis) is still in need of further investigations (AlShawaqfeh et al., 2017).

It is also thought that an altered microbiota persists after clinical improvement as this clinical recovery is not accompanied by significant changes in the fecal microbiota or in serum metabolite profiles. As so, it is believed that it can take an undetermined period after medical treatment to recuperate the standard flora (Minamoto et al., 2015).

Dietary composition changing, antibiotics, prebiotics, probiotics and TMF may be some of the approaches that can help on modulating the intestinal bacteria and consequently the inflammation. Since the differences in the intestinal microbiota and the changes resulting from the precedent approaches are extremely variable between individuals and for each dysbiosis pattern, it is still not clear what is defined as a normal and balanced intestinal ecosystem and which approach or combination of approaches are the most adequate on regulating the gut microbiota (Honneffer, 2014; Suchodolski & Allenspach, 2019; Suchodolski, 2016).

1.2.3. Environmental factors

Stress is admitted to negatively influence the homeostasis and the neuronal regulation of mucosal immune responses by increasing the release of neuropeptides. Aside from their potent capacity of modulating the immune response, they are documented to have a significant role in a variety of functions related to the GI tract – motor functioning of the gut and co-transmitters of enteric cholinergic neurons, increasing the enteric neuron excitability due to the release of acetylcholine (Wouter, 2015).

The contribution of stress as a causative, perpetuating or accentuating factor is still unclearly established in dogs. A study relating the anxiety and chronic inflammatory bowel disease in dogs aimed to compare the emotional status in healthy and IBD dogs by asking the owners to fill a screening questionnaire, reflecting in these last ones a significantly higher global score according to the severity of the disease and a chronic state of anxiety, suggesting that it may influence the disease clinical development (Reiwald, Pillonel, Villars, & Cadoré, 2013).

1.2.4 Dietary factors - Bovine Protein as an Antigen

In humans the linking between inflammatory bowel disease and dietary habits is already well established and confirmed by several epidemiological and experimental studies (Marion-Letellier, Savoye, & Ghosh, 2016).

Several human IBD patients state that specific foods worsen the symptoms and can induce a relapse (Limdi, Aggarwal, & McLaughlin, 2016; Zallot et al., 2013). A western-diet based on high dietary intakes of meat, fats, ω-6 polyunsaturated fatty acids (PUFAs), and low fruit and vegetables appears to be associated with an increased risk of Crohn's disease and ulcerative colitis (Cosnes, Gower–Rousseau, Seksik, & Cortot, 2011) by directly disturbing the mucosal immune barrier function. Also, high contents of proteins specially of animal origin have been associated with high incidence of IBD (Hou, Abraham, & El-Serag, 2011) and likely relapse (Jantchou, Morois, Clavel-Chapelon, Boutron-Ruault, & Carbonnel, 2010; Marion-Letellier et al., 2016). In mice, this type of diet also caused dysbiosis with increment of adherent-invasive *Escherichia coli* and intestinal barrier disruption (Martinez-Medina et al., 2014), supporting the hypothesis that specific diets can have modulatory effects on the intestinal microbiota and consequently triggering an immune response (Lee et al., 2015; Marion-Letellier et al., 2016).

Marion-Letellier (2016) based on several studies concerning human dietary components and IBD, reaffirmed the connection between nutrients as an etiological factor on altering the innate immune response and its significance on the pathogenesis of the disease.

Dietary antigens contact in high concentrations with the intestinal mucosa, being the mucosal surface a primary barrier against bacterial and these types of antigens. In CE dogs, a disrupted intestinal barrier resulting in increased absorption of macromolecules concurrent with a loss of immune tolerance are factors that enhance the pathogenicity of the disease by an overstimulation of the systemic antibody production. The positive clinical response to dietary changings often verified in CE dogs and the recognition of FRE has enhanced the importance of certain foods as a possible trigger of the immune-system and as a factor of etiopathogenesis.

Food specific antibody levels as anti-Bovine Serum Albumin IgA (anti-BSA IgA) and anti-Bovine Serum Albumin IgG (anti-BSA IgG), may be active intermediates on immune-mediated food intolerance. Adverse food reactions may act through non-immunologic (food intolerance) and immunologic (food hypersensitivity) mechanisms. Food contains a vast range of protein which in most cases are potentially antigenic, yet only few of them have been shown to be allergenic. An allergen is an antigen that is capable of binding to specific IgE and initiate mast cell degranulation triggering a type-I hypersensitivity (Sheldon, Wheeler, & Riches, 2014). If the allergenic protein is adequately hydrolyzed, the allergen is unable to bind to IgE and therefore incapable of triggering the mast cell degranulation (Cave, 2006). The allergens must be polyvalent, meaning they should have several epitopes that are able to bind IgE antibodies for being capable of cross-linking to the mast cell in order to initiate degranulation, therefore there is a minimum molecular weight limit on the molecules that can stimulate IgE-mediated immune reactions (Cave, 2006; Lehrer, Horner, Reese, & Taylor, 1996). As in CE dogs, the significance between an overstated immune response against food antigens and the occurrence of food intolerance in human IBD cases is not well established as well as the reason why the intensity of the antibody production is variable concerning distinct microbial and food antigens (Frehn et al., 2014).

Although several values about the limit protein size that is lower enough to avoid IgE-mediated reactions have been postulated, the most part of the studies point to 10kDa as minimum limit, since the majority of the known food allergens between 10 and 70 kDa are able to induce the production of allergen-specific IgE and to stimulate the immune response (Lehrer et al., 1996). However, a much smaller limit of 3 to 5 kDa has been suggested (van Beresteijn, Meijer, & Schmidt, 1995; Van Hoeyveld, Escalona-Monge, DE Swert, & Stevens, 1998). The upper limit is reported at 70kDa since proteins of this size are hard on passing through the enteric mucosa intact (Cave, 2006).

The antigenicity of food proteins can be reduced by disrupting the tridimensional structure of the protein, changing the aminoacidic side chains and cleaving peptide bonds (enzymatic hydrolysis) by reduction of the allergenic epitopes that causes the hypersensitivity reactions using specific proteolytic enzymes (Cave, 2006).

The lipid and carbohydrate source should also be carefully chosen as they can be also a source of allergens. However, its likely to be a protein allergen within the carbohydrate source causing the reaction than the carbohydrate source itself. Concerning a carbohydrate source like corn, it is likely to find in its composition a protein allergen such as corn zeins (Frisner, Rosendal, & Barkholt, 2000) or in lipidic sources as vegetable oils (i.e. sunflower oil), lipophilic protein allergens (Zitouni et al., 2000). Since these products do not usually undergo hydrolysis, there is a possibility that some immunogenic protein allergens can be present. Alimentary hypersensitivity/intolerance are commonly reflected in gastrointestinal and/or dermatological disorders such as pruritus (Paterson, 1995; Proverbio, Perego, Spada, & Ferro, 2010).

Although the contacted dose is probably an important factor, the intrinsic immunogenicity of the protein is the decisive factor (Lehrer et al., 1996). Bovine protein is believed to be the most common allergen in dogs (Cave, 2006) although sensitivity to chicken protein, dairy products and eggs have also been reported. The identifying of the ingredient that is triggering the immune-response often relies on trial and error. Accordingly, a strict home cooked diet with a novel protein and carbohydrate source should be tried being the owner's compliance crucial on transmitting to the clinicians the past exposure proteins and carbohydrates in order to achieve therapy success. Commercial hypoallergenic diet – hydrolyzed protein or new protein source – is also an option, although there is a relative percentage of failure related to cross-contamination in processing, partially hydrolyzed proteins or contaminants that trigger the immune response. During this period, it is not recommended to add supplements or other food components since they can also be potential sources of allergens (Gaschen & Merchant, 2011).

Elimination diet trial for at least two months may be enough for conducting which food ingredients should be avoided by following the relapsing of the clinical signs once the specific ingredient is re-introduced. Gastrointestinal clinical signs usually improve in two weeks, whereas cutaneous clinical signs may take a few weeks longer (an average of eight to twelve weeks) after starting the new diet (Gaschen & Merchant, 2011).

A dietary intake with high contents of protein affects the canine intestinal microbiota, which may lead to dysbiosis and as referred before, to trigger or enhance the gut inflammation. The majority of the commercial diets currently on sale do not have a percentage of protein as high as needed to develop dysbiosis, as high percentage of protein is related to an increment of E.*coli* and a decrease of *Faecalibacterium (*Firmicutes phylum*)* (Herstad et al., 2017; Sandri,

Dal Monego, Conte, Sgorlon, & Stefanon, 2017), well described in several articles referred previously in this study.

Cross-reactivity among food allergens have been confirmed and as so, it is important to refer, that specially for animal proteins, there are cross-reactivity allergens among various meat sources, especially in those with closer taxonomic relationship due to the high similarities of their aminoacidic sequences. Once the dog is stabilized and the offending allergens identified, the owner should be encouraged to maintain this new suitable diet for a life-long period (Gaschen & Merchant, 2011).

Although there are several evidences confirming the relation between dietary factors and the influence on pathogenesis, development, clinical response and treatment of gastrointestinal disorders, whether the inflammatory processes are a primary cause or a consequence of the followed dietary options is still in need of further studies.

1.3 CLINICAL PRESENTATIONS AND CLINICAL SCORING SYSTEMS

1.3.1 Clinical Signs

Chronic persistent or intermittent (>3weeks), more often small bowel diarrhea with vomiting are the most frequently observed clinical signs, often with dysorexia or anorexia, melena and weight loss. Hematochezia, mucus and straining feces can reveal involvement of large bowel. If both small and large intestine are affected, mixed GI signs are observed. The supra-referred signs are however unspecific. As so, complementary exams take an important role in the diagnosis and treatment of CEs.

If severe enteritis, protein-loss can occur (PLE) with consequent effusions, ascites and peripheral edema (Simmerson et al., 2014).

CE is characterized for cyclical episodes of consequent crisis and remissions which may suggest that there is no healing, but clinical management of this disease.

1.3.2 Clinical Score CIBDAI and CCECAI

Scoring systems have been important for recording the severity of clinical signs, staging the disease and for evaluating the clinical response to treatment.

In 2003, and based on an analysis of the most frequently observed clinical signs in dogs with CE, the canine inflammatory bowel disease activity index (CIBDAI) was proposed. This scoring system was shaped for evaluating the severity of the disease based on the assessment of six main clinical signs – attitude/activity, appetite, vomiting, stool consistency, stool frequency, weight loss. The cumulative score classifies the disease as clinically insignificant, mild IBD, moderate IBD and severe IBD (Jergens, 2004; Jergens et al., 2003). CIBDAI has been proved

to be useful while monitoring the course and development of the disease and treatment, although the scoring does not correlate with the observed histopathologic grade (Allenspach et al., 2007; Garcia-Sancho, Rodríguez-Franco, Sainz, Mancho, & Rodríguez, 2007; Schreiner et al., 2008) as well as being helpful on detecting early relapses (Rychlik et al., 2012).

CCECAI comes in 2007 as a most complete and accurate score clinical index, by including serum albumin concentrations, ascites, peripheral edema and pruritus parameters to the previous score system. These factors were not present in CIBDAI scoring system (Allenspach et al., 2007). Accordingly to this scoring system, the clinician is able to attribute a score based on a set of clinical and biochemical parameters, on an maximum overall possible score of 27. A score from 0 to 3 is classified as insignificant disease, from 4 to 5 as mild disease, from 6 to 8 as moderate disease, from 9 to 11 as severe disease and greater than 12 as very severe disease.

Dogs with FRE have lower scores in CCECAI systems and sub-normal to normal concentration levels when compared to ARE or IRE dogs (Allenspach et al., 2007, 2016). PLE and neoplastic clinical cases have usually highest CIBDAI score.

These scoring systems – Table 1 - allows the clinician to better describe the clinical presentation of the diseased dogs and monitoring the disease activity, therefore achieving a better method of comparison between the presentation at the time of diagnosis and after imposing a treatment. Additionally, it is also helpful on prioritizing differential diagnosis and orientating to the possible etiology.

Table 1 - *Clinical Activity Indices CIBDAI and CCECAI. Adapted from Allenspach et al. (2007) and Jergens et al. (2003)*

CIBDAI	CCECAI
○ Attitude/activity 0 - normal 1 - slightly decreased 2 – moderately decreased 3 – severely decreased ○ Appetite 0 - normal 1 - slightly decreased 2 – moderately decreased 3 – severely decreased ○ Vomiting 0 - none 1 – mild (onetime/week) 2 – moderate (two to three times/week) 3 – severe (more than three times/week) ○ Stool consistency 0 - normal 1 - slightly soft feces or fecal blood and/or mucus 2 – very soft feces 3 – watery diarrhea ○ Stool frequency 0 - normal 1 - slightly increased (two to three times/day) 2 – moderately increased (four to five times/day) 3 – severely increased (more than five times/day) ○ Weight loss 0 – none 1 – mild (<5% loss) 2 – moderate (5-10% loss) 3 – Severe (>10%loss)	CIBDAI parameters + new assessments: ○ Serum Albumin 0 – albumin >2g/dl 1 - albumin 1,5-1,9 g/dl 2 – albumin 1,2-1,4 g/dl 3 – albumin <1,2 g/dl ○ Ascites and Peripheral Edema 0 - none 1 – mild ascites or peripheral edema 2 - moderate amount of ascites/peripheral edema 3 – severe ascites/pleural effusion and peripheral edema ○ Pruritus 0 – no pruritus 1 – occasional episodes 2 – regular episodes 3 – regular episodes of itching with sleep disturbance
• Cumulative Score 0-3 – Clinically insignificant disease 4-5 – Mild IBD 6-8 – Moderate IBD >9 – Severe IBD	• Cumulative Score (CIBDAI score + new CCECAI assessments) 0-3 - Insignificant disease 4-5 - Mild disease 6-8 - Moderate disease 9-11- Severe disease >12 - Very severe disease

1.4 DIAGNOSIS

A phased clinical approach is important at the time of diagnosis. Collecting a precise clinical history and physical examination, localization of the origin of the GI clinical signs in small or large intestine such as other signs that can reveal concomitant occurring diseases are important factors. Clinicopathological testing, urinalysis and fecal analysis can help to better assess the list of differential diagnosis and exclusion of concurrent extra-gastrointestinal diseases. Diagnostic imaging, particularly abdominal ultrasound is also a valuable tool. However, all of these findings are usually non-specific, being only helpful to guide the clinician.

A clinical staging should also be achieved with the help of CCECAI scoring system and endoscopy (Allenspach et al., 2007). Since the majority of dogs do not require immunosuppressant treatment, and before advancing to more invasive methods such as endoscopy, one at a time and subsequent therapeutic essays are followed based on dietary and antimicrobial trials (Dandrieux, 2016). If the patient does not clinical respond to these two therapeutic approaches, endoscopy with biopsy sampling should be discussed to confirm presence and type of inflammation, as well as excluding neoplastic hypothesis (Simpson & Jergens, 2011).

It is important to mention that these methods should be reserved for refractory (either no response recurrence of clinical signs) cases to therapeutic trials previously referred, as histology is not able to differentiate between FRE, ARE and IRE (Allenspach et al., 2007; Luckschander et al., 2006; Schreiner et al., 2008), unless the clinician considers that the patient clinical presentation reflects that it is not capable of supporting the previous steps (i.e. PLE), or high suspicion of diffuse intestinal tumors or granulomatous colitis in predisposal breeds and that a more invasive method should be carried in advance (Dandrieux, 2016).

1.4.1 Clinicopathologic Features

Evaluation of clinicopathologic variables are easily performed but usually not diagnosis specific, however it allows the clinicians to orientate the diagnosis as well as excluding other affections (e.g. complete blood count, hemogram, measurement of the hepatic and renal parameters for assessing hepatic and renal function and exclude renal and hepatic disease, cPLI for excluding pancreatitis, serum trypsin-like immunoreactivity (TLI) for excluding exocrine pancreatic insufficiency , cortisol to differentiate Addison or hypoadrenocorticism, folate and cobalamin) in order to better have an overall feedback about the patient health status (Dandrieux, 2016; Washabau et al., 2010).

1.4.1.1 Hematologic changes and biochemical variables

A non-regenerative anemia in consequence of a chronic inflammation or blood loss can be revealed. Also, eosinophilia will often be present in cases if eosinophilic enteritis. Neutrophilia and thrombocytosis can also be decurrent from eventual erosions or ulcers in the intestinal mucosa, reflecting blood loss across the GI tract (Jergens & Simpson, 2012).

Subclinical thrombocytopenia is an uncommon observance. Also, the platelet count measured is not correlated with the severity of the intestinal inflammation and it is believed to be a consequence of the immune stimulation from the contacted antigens and with the altered immune response (Ridgway, Jergens, & Niyo, 2001). This should be taken into account when using specific immunosuppressants that can cause bone marrow suppression, aggravating the platelet level.

A recent study by Volkmann et al.(2017) confirms what other studies have already reported that anemia, hypoalbuminemia and severe hypocobalaminaemia are associated with poor prognosis (Allenspach et al., 2007) highlighting their prognostic, but not diagnostic value as they are unspecific findings.

Hypoalbuminemia is considered when albumin levels are below 20g/L and is strongly associated with a negative outcome (Craven et al., 2004; Allenspach et al., 2007) as it indicates protein-loss. The average of the serum albumin levels in SRE are, when compared to the dogs with FRE, significantly lower. This may reflect the severity of the disease in IRE dogs and some degree of protein-loss. Overall, the measurement of albumin has essentially a prognostic value (Allenspach et al., 2007; Dossin & Lavoué, 2011; Heilmann, Otoni, et al., 2014; Wennogle, Priestnall, & Webb, 2017).

Hypocobalaminaemia is often found in dogs with ARE or in dogs with exocrine pancreatic insufficiency (EPI). It is usually secondary to cobalamin ileum malabsorption or a consequence of dysbiosis and it also correlates with a poor prognosis in dogs with CE. While hypocobalaminaemia is associated with ileum lesion – where the intrinsic factor-cobalamin complex is internalized through specific receptors -, folate deficiency is due to duodenal and proximal jejunum lesions. Usually, when hypocobalaminaemia is present, low levels of albumin are also reported (Allenspach et al., 2007).

Hypovitaminosis D has been reported in both CE and PLE dogs. The serum 25(OH)D concentrations have been shown to be correlated with the severity of the clinical signs, and as so, with high levels of scores in CIBDAI (Gow et al., 2011; Titmarsh et al., 2015). Dogs with IBD and hypoalbuminemia often have hypocholesterolemia secondary to malabsorption, high parathyroid hormone and consequently low serum 25 hydroxyvitamin D concentrations with total and ionized hypocalcemia which reflects in tremors and/or seizures (Gow et al., 2011) as well as hypomagnesaemia, lymphopenia, thrombocytosis, lower serum creatinine,

hypocobalaminaemia and decreased serum α1-proteinase inhibitor fecal excretion (Allenspach et al., 2007; Equilino et al., 2015; Simmerson et al., 2014).

It is suggested that increased serum cPLI negatively affects the outcome, but the specific clinical association is still in need of additional researches.

1.4.2 Fecal examination

Fecal parasitic identification techniques such as direct wet mount or flotation techniques are used to exclude parasitic infections namely nematodes or protozoa. Fecal cultures can also be performed if there is pathogenic *Campylobacter* or *Salmonella* suspicion (Jergens & Simpson, 2012), however culture procedures implies resources that are often only available in reference laboratories, and as so in order to get results, the samples need to be previously submitted (Blagburn & Mount, 2017).

Collecting fresh fecal specimens directly recurring to a fecal loop or a thermometer is the most adequate manner of obtaining samples since it avoids secondary contaminations. However, a negative result does not allow to infer that the animal is not parasitized. Moreover, in diarrheic dogs, it is possible to result in false negatives due to the dilution effect on the total amount of parasites. Specimens collect from the ground, may also be contaminated and lead to an erratic result. If necessary, feces can be stored in an air deprived container and refrigerated for several days to a week without affecting most parasites, nonetheless *Giardia's* trophozoites and some nematodes are not capable to survive storage. As so, if in suspicion of specific parasites, the specimen must be examined immediately after collection (Blagburn & Mount, 2017).

If submitted to another laboratory for molecular or immunologic techniques as it is often needed, the specimens should be stored in formalin, in the quantities advised by the reference laboratory. Several immunological and molecular techniques have been developed and are currently used for recognizing parasites by their specific proteins or DNA. While indirect-fluorescent antibody (IFA) tests have been shown to be more accurate to identify *Giardia* spp. and *Cryptosporidium* spp. an indirect ELISA - SNAP *Giardia* - is also an adequate technique for confirming *Giardia*-induced cases of diarrhea but not for screening, since this parasite can be also found in healthy patients. Concerning molecular techniques, we can also recur to fecal PCR, as they are particularly useful on detecting parasites that are present in low number (Blagburn & Mount, 2017).

A wide spectrum anti-parasiticide, usually fenbendazole at 50mg/kg PO SID for 3-5 consecutive days, is recommended in order to cover the most common causes of parasitic infections and the intermittent character of elimination of some parasitical forms (e.g. *Giardia* spp.) (German, Hall, et al., 2003; Kent, 2017; Simpson & Jergens, 2011; Steiner, 2008).

1.4.3 Diagnostic Imaging

Abdominal radiology is not by itself a method for CE diagnosis. However, it may help to exclude extra-gastro intestinal tract disorders that can secondarily cause CE.

Ultrasound is usually the preferred imaging tool. This complementary exam is frequently used as an adjunctive tool for guiding and narrowing diagnosis hypothesis particularly in cases of GI chronic signs, and is accepted to be suitable for evaluating focal or diffuse mucosal lesions, the thickness of the intestinal layers and mesenteric lymphadenopathy or infiltrative/neoplastic disorders such as lymphoma (Gaschen, 2011; Gaschen et al., 2008). Obstructions, foreign bodies and intussusceptions can also be excluded by ultrasound examination (Kent, 2017).

There was a significant correlation between the ultrasound score and CIBDAI before treatment, but, and even despite a significantly improvement of the clinical CIBDAI score during therapy, the same was not verified in the posttreatment assessment of the same parameters. Also, the intestinal wall thickness and mucosal echogenicity are also low specific and sensitive findings (Gaschen et al., 2008).

A recent study intended to assess the diagnosis utility of abdominal ultrasound in dogs with diarrhea. In a total of 149 dogs that went through abdominal ultrasound, 44% presented no ultrasonographic abnormalities. Abdominal ultrasonography was able to identify a specific cause in only four dogs (3%), being them portosystemic shunts, linear foreign body and a perforated pyloric ulcer, therefore, any case of CE was identified by means of this complementary exam. It was also considered to have moderate utility in 38% and no utility at all in the majority of the dogs (53%). Furthermore, it was also reported to be counterproductive in 7% of the dogs, being the results of the ultrasound either falsely positive or falsely negative and leading to erratic conclusions. This study also suggests that although ultrasonography is commonly used as adjunctive exam in the diagnostic of dogs with chronic diarrhea, the results obtained are non-specific and most likely suitable to be applied to dogs with acute clinical onset. These findings questions the need for using abdominal ultrasound as a routine exam on diagnosing chronic enteropathy (Mapletoft, Allenspach, & Lamb, 2018).

1.4.4 Endoscopy/Laparoscopy with biopsy and histopathological characterization

Upper and lower GI endoscopy allows the direct visualization of the esophagus, stomach, duodenum, jejunum, ileum and colonic portions of the GI tract. Lesions such as erythema, friability, granularity and mucosal erosions and ulcerations are the most observed lesions in canine IBD (Jergens, Moore, Haynes, & Miles, 1992; Allenspach et al., 2007; Garcia-Sancho et al., 2007). Obtaining endoscopic biopsy samples is an invasive procedure, as it needs general anesthesia. This may represent a problem in CE cases with severe clinical forms of the disease, particularly PLE, and severe hypoalbuminemia (serum albumin <2g/dL) as the clinical consequences of low levels of albumin are associated with raised anesthetic risk (Gaschen et al., 2019).

As macroscopic and microscopic appearance are poorly correlated, in the presence of clinical history and signs that point to CE, endoscopy with mucosal biopsy sampling or exploratory laparotomy are often considered to characterize the inflammation according to the cells population and to exclude intestinal diffuse tumors such as lymphoma (Kenneth Simpson & Jergens, 2011). To ensure a good coverage, gastric, duodenal, ileum and cecum biopsies are usually made. The ACVIM Consensus Statement provided endoscopic, biopsy and histopathologic guidelines for the evaluation of gastrointestinal inflammation suggesting that ileal biopsies can reveal lesions not apparent in the duodenum and, therefore, sampling of this intestinal segment should be routinely performed (Washabau et al., 2010).

The observation of severe lesions in the duodenal mucosa has been shown to be significantly associated with a negative clinical outcome. This has not been verified for colon observed lesions. Therefore, endoscopic scoring of the duodenal mucosal lesions may justify the appliance of an early and more aggressive treatment (Allenspach et al., 2007).

Endoscopic examination observation with gastric and intestinal sampling and identification of the inflammatory cell infiltration type is important on confirming a diagnosis for IBD and its extension as well as ruling out neoplastic hypothesis. CE's are currently classified according to the predominant cellular infiltrate within the lamina propria together with mucosal architectural changes. Histologic examination allows the inflammatory infiltrate's predominant cell type characterization, to determine the severity of the disease and tissue morphological changes, providing several important information on the appropriate therapy plan and prognosis (Collins, 2013). Also, in cases of refractoriness to treatment (no response or relapsing), re-assessment of the diagnosis may be indicated. After gradually decreasing immunosuppressive therapy, sampling should be obtained (or reobtained) in order to rule out neoplasia, infectious disease or orienting the diagnosis (Gaschen et al., 2019).

However, histology will not differentiate the subcategorizations of CE, meaning that we cannot distinguish FRE from ARE or IRE (Dandrieux, 2016). As so, endoscopy should be reserved to the cases where alimentary changing and antibiotics have been ruled out as an effective treatment (Luckschander et al., 2006; Allenspach et al., 2007; Schreiner et al., 2008; Dandrieux, 2016; Jergens & Simpson, 2012; Washabau et al., 2010). Also, it is important to refer that not always the clinical improvement is reflected and followed by histopathological lesions (Allenspach et al., 2007; Craven et al., 2004) and that the severity of the histopathological findings and clinical score at the time diagnosis are not correlated with the long-term outcome (Allenspach et al., 2007).

1.4.4.1 Histopathology

Due to the high variability between interpretation of histopathologic findings in gastrointestinal biopsies, a group was nominated to establish guidelines to standardize the assessments of GI biopsy amongst pathologists (Day et al., 2008). In order to minimize the subjectivity and enhance the consistency of the histopathologic evaluations, the World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization Group developed standard report forms for diagnosis and treatment of gastrointestinal disease in dogs and cats (Washabau et al., 2010). A consensus on the normal histology of the GI tract was developed and then, helpful descriptions of the histopathological alterations such as morphological and inflammatory features observed in the gastric body and antrum, duodenum and colon of dogs with chronic enteropathy and concluding a final diagnosis between the different infiltration cell types (e.g. normal tissue, lymphoplasmacytic inflammatory, eosinophilic inflammatory, neutrophilic inflammatory, lymphangiectasia, mucosal atrophy/fibrosis (non-inflammatory) or other) (Day et al., 2008) well descripted in the American College of Veterinary Medicine (ACVIM) Consensus Statement on ‘‘Endoscopic, Biopsy, and Histopathologic Guidelines for the Evaluation of Gastrointestinal Inflammation in Companion Animals’ (Washabau et al., 2010). Some years later, a modification to this template was proposed, presenting a simplified histopathologic model and with the addition of new parameter – goblet cell depletion – once there is evidence that a depletion of the number of these cells may be a cause for colitis (Jergens et al., 2014). Still, several pathologists found no agreement between the template and the clinical scoring attributed especially between mild and moderate lesions consequent of the processing sampling treatment (Willard et al., 2010), which reflects the need of further studies in order to better validate the previous guidelines.

The major disadvantage concerning biopsies and histological methods reported is that the interpretation and efficacy of sampling varies massively according to the clinician, pathologist and laboratory operator (Willard et al., 2002; Willard & Mansell, 2011) as the use of non-adequate instrumentation, bad quality of method of obtention or processing, or low number of samples may have severe effects on the sensitivity for identifying lesions and obtaining a

correct diagnosis (Jergens, Willard, & Allenspach, 2016; Willard et al., 2008). Although biopsy guidelines are already set, in 2016 Jergens et al. reviewed how to maximize the diagnostic utility of flexible endoscopic biopsy in dogs and cats with gastrointestinal disease, with practical and technical considerations.

Recently, Allenspach (2018) attempted to correlate the gastrointestinal histopathologic changes to the clinical disease activity according to CIBDAI/CEECAI, proposing a novel quantitive simplified scoring system with increased utility in correlating histopathologic aspects to IBD clinical activity. This recent system includes WSAVA morphologic and inflammatory features and objective and descriptive information on the extent of GI mucosal inflammation, improving the consistency of interpretation amongst pathologists when describing the histologic lesions of the biopsy specimens (Allenspach et al., 2018).

Inflammatory infiltrates may vary on population and severity. Lymphocytes and plasma cells can characterize lymphoplasmacytic enteritis (LPE) which is the most frequent diagnosed form in dogs with CE and the typical cell infiltration associated with Immunoproliferative Enteropathy in Basenjis (Day et al., 2008; Hall & Day, 2017; Washabau et al., 2010; Wennogle et al., 2017; Willard et al., 2010).

Eosinophils in large number with concomitant eosinophilia characterizes eosinophilic infiltration (Jergens & Simpson, 2012). This finding may suggest parasitic etiology, dietary allergy/intolerance (E. Hall & Day, 2017) or Addison (Marks, 2012), revealing in the most several cases erosions or ulcers in the small intestine and gastric and/or large intestine mucosa, with clinical presentation of melena, hematemesis or hematochezia. Tendentially affects young animals and breed predisposition is reported for German Shepherds, Boxers and Dobermans (Hall & Day, 2017).

The observance of macrophages or neutrophils are rare and may lead to the suspicion of an infectious process specially in Boxers of French Bulldogs (Granulomatous colitis or Boxer's histiocytic ulcerative colitis), which may indicate the realization of FISH, culture or cytology with histopathological analysis, special stains such as PAS or Gram for detecting the agents. Infectious agents may be bacteria (e.g. *Escherichia coli*, *Streptococcus*, *Campylobacter*, *Yersinia* and *Mycobacteria*) or fungi's (e.g. *Histoplasma)* (Craven et al., 2011; Jergens & Simpson, 2012; Manchester et al., 2013; Mansfield et al., 2009; Simpson et al., 2006).

Architectural changes may include villus morphology alterations such as stunting (hypoplasia/aplasia), crypt abscesses and dilatation, villus lymphatic dilatation, goblet cell mucus content and fibrosis (Dossin & Lavoué, 2011; Forman, 2016; Peterson & Willard, 2003; Simmerson et al., 2014). Animals with PLE often present these histopathological architectural changes. Although severe morphologic alterations were observed in the intestinal biopsy samples in dogs with hypoalbuminemia when compared to the normoalbuminemic dogs, there

is no significant difference between the lymphoplasmacytic and eosinophilic cellular infiltrate proportions among the two groups (Wennogle et al., 2017). Additionally, the severity of the infiltration was not significantly correlated with the measurements of the serum albumin, confirming what previous studies had already stated (Allenspach et al., 2007).

It is important to refer that there is no significant correlation between histological score from the endoscopic biopsy specimens of intestinal inflammation and the clinical score at the time of diagnosis or after treatment (Jergens et al., 2003; Washabau et al., 2010).

Endoscopic and histopathological evaluation of the intestinal biopsy samples is still the gold standard for characterizing and quantification of the intestinal inflammation and thus for CE diagnosis, specially the IBD form.

1.4.5 What is a biomarker? Biologic Inflammatory Markers

A biomarker is defined as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention', which may reflect diagnostic, staging, clinical indicator, predictor or monitoring importance (Atkinson, 2001). The ideal biomarker must allow, according to Jergens et al., to identify patients at risk of developing the disease, be specific, easy to perform, be able to detect or follow the disease or its response to treatment, it should permit serial evaluation and have predictive value of relapsing or recurrence (Jergens & Simpson, 2012). In the last few years, efforts have been made in order to find specific serological and laboratory markers that could help in the early detection and assessment of gastrointestinal inflammation. Serum markers, if capable of predicting sub-clinical inflammation, may become a promising and important non-invasive weapon of diagnosis even though until now it is still not known one that is specific for chronic enteropathies. Biomarkers that can help on defining the presence of disease, the severity, the response to treatment and monitoring would be clinically helpful in CE dogs. Despite the variability of biomarkers available, long-termed studies are needed in order to correctly evaluate the utility of the biomarkers in clinical environment related to CE dogs (Heilmann & Steiner, 2018) as the ideal parameters are still not established. At the present time, biomarkers are likely useful as auxiliary parameters on building an overall look about the patient health status, together with the other diagnosis tools.

1.4.5.1 C-Reactive Protein

C-Reactive Protein (CRP) is an acute phase reactant protein, marker of systemic inflammation, that is synthetized by the liver, and rises massively and quickly when in present of an inflammatory stimulus. Its relevance as a non-invasive biomarker is recognized in both veterinary and human medicine (Dubinsky, 2010; Nakamura et al., 2008; Otoni et al., 2018) and that is severely increased in human IBD, working as an inflammatory biomarker.

CRP levels have been confirmed in several studies to be increased in canine idiopathic IBD (Jergens et al., 2010; Jergens et al., 2003; McCann, Ridyard, Else, & Simpson, 2007; Otoni et al., 2018). However it is not specific for IBD form or even CEs, showing increment of its values in other inflammatory processes and limiting its clinical usefulness as a specific biomarker (Nakamura et al., 2008). Nonetheless it may be useful on monitoring the response to treatment, since an improvement of the inflammatory status would be accompanied by a decrease of the serum CRP values (Collins, 2013).

This acute phase protein was thought to be particularly important during the disease monitorization process, as a decreasing of serum CRP concentrations was correlated with lowering of the clinical score in response to an effective dietary or medical therapy. As so, lowered levels of CRP could be a reflection of a good clinical response to treatment (Jergens et al., 2010). Although some studies demonstrated that there is a lack of correlation between CRP concentrations and the clinical score or histopathological findings (McCann et al., 2007; Otoni et al., 2018), a recent study brought interesting data since it reported that serum CRP levels superior to 9.1mg/L are able to distinguish IRE dogs from FRE/ARE with a 72% sensitivity and a 100% specificity (Heilmann et al., 2018).

1.4.5.2 ANCA (Antineutrophil cytoplasmic antibodies)

Anti-neutrophil cytoplasmatic antibodies (ANCAs) are autoantibodies mainly of IgG type, directed against antigens found in the cytoplasmic granules of neutrophils and monocytes. According to the position of the antigenic-antibody complex, visualized by indirect immunofluorescence, there are two main types of ANCA - cytoplasmic-antineutrophil cytoplasmic antibodies (cANCA) and perinuclear-antineutrophil cytoplasmic antibodies (pANCA) (Allenspach et al., 2004; Dasgupta & Wahed, 2014; Florey et al., 2017; Luckschander et al., 2006; Mancho et al., 2010; Otoni et al., 2018).

A study meant to assess the clinical value of perinuclear antineutrophilic cytoplasmic as a serologic marker of canine IBD. The pANCA indirect immunofluorescence (IIF) assay revealed high specificity (between 83 and 95%), but a 51% sensibility for canine IBD which discredited its suitable use (Allenspach et al., 2004). A later study aimed to verify the correlation between pANCA and CIBDAI, endoscopy and histopathologic clinical scores before and after treatment in FRE and IBD dogs, finding no correlation between this biomarker values before and after treatment (for both FRE and IBD dogs) and the parameters evaluated, exceptuating the endoscopic duodenal score in FRE dogs after treatment. This finding supports that a positive pANCA test before therapy may be a helpful marker on verifying a good clinical response to therapy in FRE group as the endoscopic duodenum score after treatment was significantly correlated to pANCA titer measured before treatment (Luckschander et al., 2006). In 2010,

results indicated that pANCAs are high specific markers for differentiating IBD dogs from other digestive disorders (Mancho et al., 2010).

In 2017, a study tried to evaluate if a commercially available granulocyte indirect immunofluorescence assay designed for humans could be used for detection of serum pANCA in CE dogs as the previously used IIF were lengthy procedures and interpretation operator-subjective, resulting in high variability. The two methods were in agreement of results, concluding that the newly tested assay was also appropriate. It was also identified a significant correlation between the measures of pANCA or cANCA and the diagnosis of FRE dogs, reflecting that this method could be helpful on discriminate FRE from IRE or healthy dogs (Florey et al., 2017). All of these studies had a high specificity but a low sensibility, being more useful as a confirmatory test rather than a screening test.

1.4.5.3 Calgranulin C (S100A12)

Calgranulin C (S100A12) is a marker of neutrophilic inflammation, that has been studied due to its potential role in innate and acquired immune responses to endogenous microbiota and diet antigens (Heilmann, Lanerie, et al., 2011) and is shown to be increased in serum and fecal samples of IBD dogs.

S100A12 is an endogenous damage-associated molecular pattern (DAMP) calcium-binding protein associated with the phagocyte activation. It is mainly expressed and secreted by activated neutrophils, macrophages and monocytes (Meijer et al., 2014; Vogl et al., 1999) and once released binds to serum soluble Receptor for Advanced Glycation End Products (sRAGE), a pattern-recognition receptor, creating complexes capable of signaling and activation of nuclear factor NF-kB and the amplification and perpetuating the inflammatory response (Foell, Wittkowski, Vogl, & Roth, 2007; Hanifeh et al., 2015, 2018; Heilmann, 2015; Heilmann, Otoni, et al., 2014; Hofmann et al., 1999).

S100A12 is reported to be increased in serum and fecal samples of IBD dogs and fecal S100A12 was associated with clinical severity of disease, endoscopic lesions, histologic inflammatory lesions and negative outcome (Heilmann, Grellet, et al., 2014; Heilmann et al., 2019; Heilmann, Otoni, et al., 2014) and it is not influenced by corticosteroid therapy.

Two years later, the same author developed a study that measured fecal S100A12 concentrations at the time of diagnosis between the different subcategorizations of CE (FRE, ARE, IRE and NRE), confirming in the IRE dogs a more increased value in comparison to FRE/ARE dogs although it did not permit to distinguish between them. Also, NRE dogs had significant higher fecal S100A12 values (>2700 ng/g) in comparison to dogs that had complete or partial remission with a 100% sensibility and 76% specificity, suggesting that fecal S100A12 measurement may have prognostic interest on predicting the lack of response to treatment in CE dogs (Heilmann et al., 2016).

1.4.5.4 Calprotectin

As the previous biomarker, Calprotein (S100A8/A9) is a protein complex with proinflammatory effects, that also belongs to the S100 proteins of damage-associated molecular pattern (DAMP) and that are commonly found in high concentrations in inflamed tissues, where neutrophils and monocytes are highly represented (Foell et al., 2007).

Calprotectin (cCP) is a biomarker of neutrophilic inflammation and is shown to be increased in serum and feces samples in inflammatory conditions, such as CE dogs. Calprotectin is a stable neutrophil protein complex present in the stools (Heilmann et al., 2018; Heilmann, Suchodolski, & Steiner, 2008) and plasma that when in the presence of inflammation such as human IBD or infection is severely increased (Dubinsky, 2010). The same increasing was observed in IBD dogs, as well as the decreasing of it related to a positive clinical response to treatment (Grellet et al., 2013; Otoni et al., 2018), This recent studies also verified a positive correlation between the cCP values, the CCECAI score and the histologic evidence of intestinal mucosal changes.

According to Heilmann, serum ccP may be useful on detecting inflammation in dogs, though its values seem to be increased by glucocorticoids such as prednisolone, invalidating its clinical usefulness after glucocorticoids treatment in SRE and idiopathic IBD patients specially when comparing to calgranulin C (Heilmann et al., 2012). Equilino et al., (2015) also evaluated the concentrations of several serum biomarkers in dogs with PLE, concluding that serum calprotectin concentration is not useful on differentiating FRE dogs from PLE dogs, and emphasizing that even though the values cCP are increased in overall CE dogs, serum cCP should not be considered for subcategorization the CE.

A recently developed study has confirmed that dogs with IRE have higher cCP concentrations (median 2.0 µg/g) than FRE/ARE dogs (median 1.4 µg/g) and that within the IRE group, dogs with no clinical response or partially clinical response had higher values (median 37.0 µg/g) than dogs with clinical response (median 1.6 µg/g). Although, the differences observed were not significantly different, the author confirmed a cutoff - a fecal concentration of cCP equal or superior to 15.2 µg/g - for differentiating partial responders or non-responders IRE dogs from complete clinical remission IRE dogs in two groups, with a 75% specificity probably reflecting a clinical utility on predicting the response to treatment (Heilmann et al., 2018).

The concomitant measure of fecal cCP with serum CRP or both can increase the accuracy of the diagnosis (Heilmann et al., 2018).

1.4.5.5 α1–Proteinase Inhibitor (α1-PI)

Serum α1–Proteinase Inhibitor (α1-PI) is a major plasma proteinase inhibitor that is synthetized in the liver and as it is resistant to hydrolysis, it is possible to measure its levels in the stools, revealing interest as a fecal biomarker. As a consequence of leakage, secondary to gastrointestinal inflammatory processes, proteins can be lost through the intestinal mucosa. Since it has a similar molecular weight as albumin, it was expected that canine α1-PI is lost at the same rate, together with prothrombin III and other plasma proteins that have lower molecular weight and therefore leaking earlier, being present in protein-loss cases (Dossin & Lavoué, 2011; Heilmann, Paddock, et al., 2011; Murphy et al., 2003; Willard, 2015). However, one study evaluated if fecal α1-PI values were correlated with the serum albumin concentrations in dogs with GI disorders and concluded that there was not significance correlation between these two parameters (Murphy et al., 2003). Hypotheses are that α1-PI is excreted at an earlier state of disease before albumin, when the disease is less severe.

Since protein-loss is a common factor among several disorders, it is not specific for canine CE therefore it can only be helpful on tracing the disease progression and response to therapy (Collins, 2013) and since increased fecal and lower serum α1–proteinase inhibitor (α1-PI), are common findings in CE dogs (Gow et al., 2011; M. Willard, 2015), it is expected to lower its fecal values with the amelioration of the intestinal mucosa integrity enhancing its prognostic value. Measurement of α1-PI is an uncommon performed test due to its difficult interpretation and access, although it would be a useful marker for early detection of protein-loss since the levels of serum α1-PI seems to decrease before albumin and therefore, before the clinical onset (Murphy et al., 2003).

1.4.6 Differential Diagnosis

As already discussed, IBD in dogs is a diagnosis of exclusion. For that and in order to achieve a correct diagnosis, other enteric chronic inflammation causes should be ruled out by assessing GI clinical signs and their duration, laboratory testing and complementary exams.

Elimination diet, fecal examination and culture, complete blood count (CBC), serum biochemistry with evaluation of the renal and hepatic parameters, TLI, PLI, T4, cobalamin, basal cortisol, diagnostic imaging, endoscopic or laparotomy with confirming histologically samples are part of the data that should be evaluated (Jergens & Simpson, 2012).

Infectious and parasitic agents (e.g. *Giardia*, *Trichuris*, *Ancylostoma,* enteric pathogenic bacteria), extra-digestive disorders (i.e. chronic kidney disease, chronic liver disease, chronic pancreatitis, IPE and atypical hypoadrenocorticism), lymphangiectasia, chronic intussusceptions and neoplasia such as lymphoma or mastocytoma, should be discarded (Jergens & Simpson, 2012). Also, in order to diagnose canine IBD, it implies that diet and

antibiotic trials have failed, inflammation has been demonstrated and immunosuppressants will be likely necessary (Dandrieux, 2016).

1.5 MANAGEMENT AND TREATMENT OF THE DIFFERENT TYPES OF CHRONIC ENTEROPATHIES

The main goal in CE dogs is the management of clinical signs associated to the underlying cause and its complications. Therapeutical approach is also focused on minimizing the inflammation and intestinal dysbiosis, as well as improving the albumin serum concentration levels and nutrient deficiencies as cobalamin and folates (Forman, 2016).

When treating dogs with CE, sequenced trials are used being dietary changing a first-line therapy option. As referred before, trials with diet changing and antibiotics or their combination should be attempted before advancing to minimal invading procedures such as endoscopy or exploratory laparotomy. An exception to this rule contemplates animals with low concentration levels of albumin reflecting PLE, suspicion of granulomatosis colitis in predisposal breeds or neoplasia such as lymphoma (Dandrieux, 2016).

In mild to moderate disease cases, the therapeutical method often preferred is a sequenced step approach - dietary and antimicrobial trials first, and if no clinical response, use of immunosuppressants such as prednisone or prednisolone (Dandrieux, 2016). For moderate to severe clinical cases, a coexisting treatment of dietary changing, antimicrobial use and immunomodulators is often followed (Simpson & Jergens, 2011).

1.5.1 Chronic Enteropathies without hypoalbuminemia

As mentioned above, CE are frequently subcategorized according to their treatment response in FRE, ARE, IRE. To cases that are refractory to the imposed treatment, the term NRE is used. To dogs with CE who have subnormal level concentrations of albumin, PLE is also used.

All things considered, according to the age, breed predisposition, history and clinical severity of the disease, the levels of serum albumin and cobalamin, endoscopic and histopathologic and architectural mucosal alterations and the type and severity of the cellular infiltrate different approaches are taken (Jergens et al., 2014; Wennogle et al., 2017). When there are normal serum albumin levels reflecting mild to moderate disease, the usual treatment embodies sequent procedures starting from empirical treatment with wide spectrum anti-parasiticides (fenbendazole at 50mg/kg PO SID for 3 days), an exclusion dietary trial with the minimum duration of two weeks to four weeks, antibiotic trial with metronidazole or tylosin until intestinal biopsy and/or immunosuppressant-drugs. In refractory cases or when moderate to severe disease is observed regarding the levels of serum albumin, a more aggressive approach is

preferred going from intestinal endoscopy with biopsy, empirical treatment with fenbendazole and concurrent multimodal therapy with dietary trial, antibiotics and immunosuppressants.

1.5.1.1. Dietary Therapy

Diet-changing approach is the first-line treatment in dogs, as the majority – commonly over 50% - of the cases with mild to moderate disease respond to diet alone (Allenspach et al., 2016; Mandigers, Biourge, Van Den Ingh, Ankringa, & German, 2010). The aim of dietary adjustment is to reduce antigenicity trigger factors of the immune-response, in order to minimize the intestinal inflammation (Rudinsky, Rowe, & Parker, 2018).

There are two clinical approaches concerning elimination diet alone that are commonly used based on a hyper digestible hypoallergenic diet with a as low as possible fat content. One being hydrolyzed protein diet - Royal Canin® Hypoallergenic, Hill's Prescription Diet® z/d®, Nestlé-Purina HA Hypoallergenic® - and the other on the usage of a novel commercial– Hill's Prescription Diet® d/d and Purina® Pro Plan® Veterinary Diets Canine DRM Dermatosis or Royal Canin Anallergenic™ - or home-made novel protein source to which the affected dog has never contacted before.

Hydrolyzed protein diets consists in breaking proteins into smaller peptides to reduce their molecular weight, lowering the potential of triggering an immune response to the dietary antigen (Cave, 2006; Peterson & Willard, 2003; Poulin, 2016). The aim is then to disrupt the protein structure in order to remove any existing allergens and allergenic epitopes, avoiding the immune recognition by patients that are already susceptible to the integral protein and through the same mechanism of disruption, to break the protein structure to a much smaller extent so that there are no antigens capable of triggering an immune response, consequentially leading to a sensitization in a naïve individual (Cave, 2006). Protein hydrolysis should avoid the allergen to bind to specific IgE as it would occur with an intact protein, and prevent mast cell degranulation, avoiding the immune reaction (Galli & Tsai, 2012). Therefore, hypoallergenic commercial formulas have lower potential to cause immune responses due to their substantial reduction in antigenicity. Furthermore, it would be expected that the hydrolysis process would lower the food palatability, however, studies involving chicken-based and soy-based hydrolysate diet registered in the most of the dogs good or excellent palatability or low refusal rates, completing feeding trials with this prescript diets (Biourge, Fontaine, & Vroom, 2004; Loeffler, Lloyd, Bond, Pfeiffer, & Kim, 2004).

The selection of a commercial hydrolyzed protein diet should be based on the protein source. Even taking into consideration that these formulas are hydrolyzed, the chosen diet should contain a protein source to which it is known that the animal is not sensitive or suspected to be, since hydrolysis does not guarantee the complete absence of immunogenic components. Additionally, the carbohydrates and lipid sources should also be carefully chosen since they

could also be or contain protein extracts that are a source of antigenicity (Cave, 2006; Frisner et al., 2000; Zitouni et al., 2000).

Another possibility is testing a novel antigen home-cooked or commercial diet. This is based on trying a new kind of protein or carbohydrate. However, with the reduced compliance and constant growing of the commercial dietary options, it is difficult to find a protein source to which the animal had never contacted before, reason why sometimes it is easier to start a commercial hydrolyzed protein when the owner is not sure about the dietary history of the animal or reveals contact with several proteins (Cave, 2006). In an ancient study involving dogs with lymphocytic-plasmocytic colitis submitted to a novel protein diet, it was reported that all of the patients had resolution of clinical signs. Additionally, when re-testing for their before-treatment diet, almost all of them relapse (Nelson, Stookey, & Kazacos, 1988), reflecting the importance of the owner compliance on maintaining this new diet life-long termed.

Hydrolyzed or antigen-restricted/novel protein options as first-line diet therapies are related to a very positive long-term response - more than 3 years of monitorization (Allenspach et al., 2007; Mandigers et al., 2010). In 2016, Allenspach's study spotted that there was not significantly difference in the clinical responses between the usage of hydrolyzed diet or novel antigen, showing good outcomes for the both approaches.

Many clinical studies consent that dietary changing by itself result in an evident clinical response (Allenspach et al. 2007, 2016, Mandigers et al. 2010) in a matter of a few days to two weeks. Studies encourage to maintain this new diet at least for 12 weeks after the diagnosis (Allenspach et al., 2007, 2016).

Additionally, it has been observed duodenal brush border significant healing at 6 weeks of treatment with an hypoallergenic commercial diet and decreasing of the mononuclear cell score, although the histological score remained the same (Walker et al., 2013). This last element comes to confirm previous studies (Allenspach et al., 2007; Garcia-Sancho et al., 2007; Schreiner et al., 2008). Histologically, there is tendency to observe a higher number of eosinophils and lymphocytes, but is not a FRE specific observance, reason why as referred before, dietary trial precedes always the usage of more invasive methods such as endoscopy and sampling (Allenspach et al., 2016; Dandrieux, 2016; Hall & Day, 2017).

Dietary n3:n6 fatty-acid ratio may also be a method of reducing the production of pro-inflammatory metabolites by immune-modulation (Ontsouka et al., 2010).

Feeding in divided meals has been reported to reduce the load on an already compromised intestine. As there are often relapses and there are still no effective methods to determine whether they're going to happen, the clinicians should encourage the owners to maintain this new diet (Dandrieux, 2016) even though there are studies which report the possibility to

resume, after 12 weeks, their original diet without developing clinical signs of GE (Allenspach et al., 2016; Luckschander et al., 2006; Mandigers et al., 2010).

As so and accordingly to the several previous developed studies, diet-changing alone – elimination/hydrolysate - is acknowledged to be the first-choice strategy for short and long-term as the majority of the diseased dogs will positively respond by stabilizing their clinical presentation and revealing mucosal brush border healing in just a few days. In PLE dogs, diet-changing alone can be risky regarding their guarded prognosis, reason why in these cases more invasive approaches are preferred.

1.5.1.2 Antibiotics

Effects derived from the use of antibiotics result from the combination of decreases in total bacterial quantities, changes in specific bacterial groups and indirect effect in inflammatory cytokine profiles (Suchodolski & Allenspach, 2019).

ARE characterizes the cases that have undergone two weeks of elimination diet trials and have failed to clinically respond or that are currently following one but have concomitantly better responded to empiric antibiotic treatment such as metronidazole (10-20mg/kg PO BID or TID), oxytetracycline (10-20mg/kg PO TID) or tylosin (20 mg/kg PO BID or TID) within two weeks after initiation of antibiotic therapy (Allenspach et al., 2016; Hall & Day, 2017; Westermarck, Skrzypczak, et al., 2005). The treatment is regularly prolongated for 4-6 weeks and relapses with diarrhea are commonly reported (German, Day, et al., 2003; Westermarck, Skrzypczak, et al., 2005). There are also two studies in 2011 and 2014 reporting the use of tylosin, and one of them referring that a low dose of tylosin such as 5mg/kg every 24h for seven days when relapsing can be effective on controlling the clinical signs (Kilpinen et al., 2011; Kilpinen, Spillmann, & Westermarck, 2014). The same was assessed for a 2mg/kg BID doses of metronidazole (Suchodolski & Allenspach, 2019). Although these doses seem to be capable of controlling the diarrhea, it is questionable whether they're equally efficient concerning its antimicrobial effect in the same low-doses suggested (Suchodolski & Allenspach, 2019).

These drugs are beneficial helping on balancing the intestinal flora and by their capacity on modulating the immune-response. Metronidazole has been proved to rise the number of *Bifidobacterium* and to reduce the colonic oxidative damage to proteins (Vasquez, Suau, Magne, Pochart, & Pelissier, 2009). Tylosin, is also reported to have a strong influence on the increasing of the fecal count of *Enterococcus spp.* which have anti-inflammatory proprieties and seem to be capable of modulating the stool consistency. Some of these strains have also probiotic properties and look like an alternative long-term approach (Kilpinen, Rantala, Spillmann, Björkroth, & Westermarck, 2015). There are also studies that report that tylosin simultaneously with diet-changing can have advantageous results in the clinical response of ARE dogs (Westermarck, Frias, & Skrzypezak, 2005). On the other hand, the use of antibiotics

can cause several alterations on the intestinal microbiome with consequent alterations on the metabolic pathways such as bile acid metabolism and oxidative stress which then prompts GI clinical signs, and that antibiotic administration ending also led to the cessation of the diarrhea (Suchodolski et al., 2009; Suchodolski et al., 2016).

Granulomatosis Colitis of Boxer, also reported in French and English Bulldogs and some other breeds, is the only chronic enteropathy that is confirmed to have bacterial etiology and that clinical remission can be achieved using enrofloxacin by eradication of invasive intramucosal *Escherichia coli*. Histopathology of colonic biopsies showing accumulation of macrophages with abundant eosinophilic granular cytoplasm, in a positive reaction to periodic acid-Schiff (PAS) staining material, are consistent findings to this disease. Fluoresce in situ hybridization (FISH) is a recently developed and high sensitivity technique that identifies invasive bacteria in tissues (Dandrieux, 2016) and clinical remission can be achieved by eradication of invasive intramucosal *Escherichia coli* using enrofloxacin for minimal 6–8 weeks, in daily doses of 5–10 mg/kg (Mansfield et al., 2009). In fact in 2013, (Lechowski et al., 2013) has published guidelines for the usage of enrofloxacin in canine colitis.

While in these cases the use of antibiotic is well described, in other GI clinical cases is reported to exacerbate the dysbiosis (Suchodolski, 2016). Antimicrobials may have a profound impact on the gut microbiota inducing in consequence a long-term dysbiosis which will induce by itself diarrhea in these dogs (Gaschen et al., 2019; Olson et al., 2015; Suchodolski & Allenspach, 2019). Also, their discontinuation is often related with relapsing of the clinical signs (Allenspach et al., 2016; German, Day, et al., 2003; Westermarck, Frias, et al., 2005). These findings questions the use of antimicrobials in cases of chronic diarrhea (Suchodolski & Allenspach, 2019).

All things considered, and apart from Granulomatosis Colitis of Boxes, the usage of antibiotics is still dubious and seems to be a short-timed effective approach. Allenspach et al., (2016) reports in a recent study considering the one year follow-up of 203 dogs, that between 6 to 12 months after initiating metronidazole at the doses of 15mg/kg BID, all of the animals included in the mentioned study had relapsed and that ARE dogs when compared to FRE and IRE, had worst clinical outcome while the majority of the IRE group sustained in clinical remission. This stresses the need of using antibiotics for a long period of time (Nitzan, 2016), as well as the antibiotic resistance emerging problems (Craven et al., 2010).

If the patient is clinically stable and having a good response to diet-changing and antibiotic trial, it should be continued for at least 2-4 weeks in order to confirm the diagnosis (Willard, 2016) and thereafter gradually decrease the antibiotic doses by 25% in periods of two weeks (Jergens & Simpson, 2012). If the animal does not show a positive clinical response to the antibiotic trial within 2 weeks or have a relapse a few days after stopping the antibiotic,

adjunctive therapy with immunosuppressant drugs should be considered as there are studies that report that ARE dogs when relapsing, may need immunosuppressant drugs for their life period (Hall & Day, 2017). If the relapse occurs after two months, Willard et al. (2016) defend that antibiotic should also be handed when symptomatic, however this is a topic of current interest and discussion guarding the emergent problems already referred such as higher percentage of relapsing in the ARE group in comparison to the others CE groups and antibiotic resistance related to the long-term use of sub-therapeutical doses of antimicrobials (Suchodolski & Allenspach, 2019).

1.5.1.3 Immunosuppressants

Immunosuppressants can be used as a primary or adjuvant therapeutic approach for CE. The aim of using immunosuppressants is to modulate the immune-response and helping on the reduction of the inflammation within the GI tract in order to stabilize their clinical presentation, usually with a concurrent adequate diet. The majority of these medications have side effects with GI disturbance, reason why anti-acids or intestinal motility medication should be given simultaneously (Hall et al., 2017).

IRE refers to those dogs that were refractory to diet changing and antibiotic therapy, requiring medical treatment based on individual or combinations of immune-suppressant drugs for PLE dogs or severe CE cases (e.g. prednisone, prednisolone, cyclosporine and azathioprine treatment) (Allenspach et al., 2016). Currently, there are not scientific fundaments capable of supporting the choice of a particular immune-suppressive treatment based on the specific histopathologic findings (Makielski, Cullen, O'Connor, & Jergens, 2018), being the treatment planed in accordance to the personal and collective experience of the clinician and peers (Gaschen et al., 2019).

Oral prednisolone, due to its high efficacity and low cost, is the first immunosuppressive drugs and is usually started at a higher dose of 1-2mg/kg in one or two daily doses for two to four weeks (Forman, 2016; E. Hall & Day, 2017; Jergens & Simpson, 2012; Kent, 2017; Simpson & Jergens, 2011). A checkup monitoring should be done after the first two weeks and if controlled, maintained for two more weeks. According to the response, the dose can slowly be reduced for that point on.

It is likely that some IRE dogs may need to continue a long-term corticosteroid therapy. As so, the minimal effective dose must be assessed in order to avoid secondary effects (Poulin, 2016). It is important to emphasize the importance of a close monitoring in the following weeks in order to foresee consequences such as bone marrow suppression (i.e. azathioprine), or iatrogenic hyperadrenocorticism when using specific immune-suppressants or in inadequate doses. Clinical signs such as polyuria/polydipsia, muscle dystrophy, increased appetite, poor hair coat, weakness or lethargy should warn the clinician that the dose should be adjusted.

Additionally, the susceptibility to infections is increased related to corticotherapy (Malewska, Rychlik, Nieradka, & Kander, 2011; Pietra et al., 2013), which may justify frequent routine exams.

Prednisolone is often used for short-term control in a high number of cases. However, 52% of dogs do not respond to this immune-suppressant (Allenspach et al., 2007). Cyclosporine is reported to be adequate for dogs refractory to this first corticotherapy approach (Allenspach, Rüfenacht, et al., 2006; Dandrieux, Noble, Scase, Cripps, & German, 2013; Dandrieux, 2016).

In alternative to prednisolone, a glucocorticoid called budesonide can be used. It has minimal systemic absorption, weak mineralocorticoid effects and a study reports effective remission of the clinical signs over 6 weeks of treatment. The usual doses are 2 to 3mg/kg SID, regarding they're under or over 18kg respectively (Stafford, 2017).

However, both prednisolone and budesonide when compared showed no significant difference between the incidence of adverse effects and clinical success as well as effective remission rates, over a 6 weeks trial for SRE dogs (Dye, Diehl, Wheeler, & Westfall, 2013). Additionally, Pietra et al., (2013) made efforts in order to evaluate plasma concentrations and therapeutic effects of budenoside in a 30 days trial assay and concluded that there was an adequate therapeutic response without adverse effects. However in Rychlik's study (2016) there were registered opposite results – budesonide did not reflect effective treatment by ameliorating the clinical signs, didn´t lower the CIBDAI score or improved macroscopic appearance (Rychlik, Kołodziejska-Sawerska, Nowicki, & Szweda, 2016).

Allenspach (2006) published a study about the expression of P-glycoprotein (p-gp) in lamina propria lymphocytes of duodenal biopsy samples (Allenspach, Bergman, et al., 2006). This protein is a transmembrane protein that as a drug-efflux role in the intestinal epithelium. In humans, this protein is upregulated when there is IBD refractory to steroids. In this study, low p-gp scores before initiation of steroid treatment was significantly associated with a positive response to treatment in steroid-responsive enteropathy (SRE) dogs - a subgroup of IRE, that characterizes dogs that respond to prednisolone (steroids) (Dandrieux, 2016) - meaning that the measurement of the mucosal expression of p-gp may become a predictive factor of the clinical response to steroid treatment (Allenspach, Bergman, et al., 2006).

Particularly for refractory cases or as adjunctive drugs and to lower glucorticoids doses (Simpson & Jergens, 2011), the clinician can also use ciclosporin (5mg/kg SID or BID), azathioprine (2 mg/kg SID for 3 weeks) or chlorambucil (2-6mg/m^2 SID). By this, the undesired secondary effects consequent of the usage of long-term corticoids, such as polyuria and polydipsia among others, can be better controlled.

Cyclosporin is an immunosuppressant cyclic polypeptide molecule with strong immunomodulating and immunosuppressive properties which mechanism of action relies on blocking the activation of T-cells (Faulds, Goa, & Benfield, 1993) particularly used for immune-mediated diseases, inflammatory bowel disease and dermatological conditions (e.g. atopic dermatitis and pemphigus) as well as for reducing transplants rejections (Stafford, 2017). Cyclosporin acts by specifically binding to an intracellular protein called cyclophilin-1, which forms a complex that inhibits calcineurin, that is an enzyme that prevents the activation of the nuclear translocation of activated T-cells factor (NF-AT) responsible for the production of pro-inflammatory cytokines such as IL-2, IL-4, INF-γ and TNF-α. (Guaguere, Steffan, & Olivry, 2004). IL-2 is particularly important for cellular immunity. NF-AT is also related to a regulated homeostasis and contributes for innate immunity response (Fric et al., 2012). This drug also affects the inflammatory response by lowering it, inhibits the growth and activation of B cells which affects humoral immunity, reduces the number of antigen presenting cells (APCs) and mast cells, impedes basophiles and eosinophils degranulation, reduces adhesion molecule expression in endothelial cells and as anti-proliferative effect on keratinocytes (Forsythe & Paterson, 2014). Allenspach et al. (2006) developed a study about the 'Clinical Efficacy of Cyclosporine Treatment in steroid-refractory IBD dogs', with positive results on the clinical presentation. In 2007, the same author tried a second prospective treatment trial with less results. The efficacity of the usage of this molecule in refractory cases is still in need of further studies.

Azathioprine is also an immunosuppressive and more economic option in comparison to cyclosporin that is indicated for severe and refractory canine inflammatory bowel disease (Marks, 1998). Azathioprine is an imidazolyl that relies on its cytotoxic property, which when metabolized into 6-mercaptopurine blocks purine metabolism and the antigenic triggering of lymphocytes (Jergens, 2002). Clinical improvement may only be observed after a 3-4 weeks period (Dandrieux et al., 2013). Initially the dose recommended is 2 mg/kg SID for 3 weeks, which can be reduced to 1-2mg/kg every two days (Gaschen, 2011; Hall & Day, 2017; Westermarck, 2016). Plumb (2018) reports as adverse effects when in long-term using such as myelotoxicity, thrombocytopenia, poor hair growth, acute pancreatitis and hepatotoxicity can also be associated which justifies monthly monitorization of the hematological and biochemical parameters. In 2013, it was suggested that this drug could worsen the clinical outcome – a combination of prednisolone and chlorambucil instead of azathioprine and prednisolone, in addition to an hydrolyzed diet, was then reported as being more effective for PLE cases (Dandrieux et al., 2013).

Chlorambucil is one of the most utilized second-line therapy options, usually at 2-6mg/m^2 daily doses (Stafford, 2017). It has been historically less expensive than cyclosporine and has immunosuppressant and antineoplastic proprieties, being also used for small-cell lymphoma

in cats. A study reporting positive response to this immune-suppressant in dogs raised the question that a proportion of PLE cases could possibly have small-cell lymphoma instead of chronic enteropathy. Further studies are needed in order to evaluate this hypothesis (Nakashima et al., 2015) . As azathioprine, clinical improvement may only be observed after a 3-4 weeks period, reason why the other corticosteroids should be continued during this initiating period of time (Dandrieux et al., 2013) .

Another immunosuppressant is mycophenolate mofetil, that has been target of study and growing attention in veterinary medicine. Although it seems to be potentially useful, there is still no information about safety and adverse effects in dogs or efficacy. This pharmaceutical drug acts by decreasing the production of nitric oxide and by inhibiting the synthesis of inosine monophosphate dehydrogenase, that limits the purine synthesis of guanosine nucleotides. As guanosine are crucial for T and B-cells, their proliferation becomes limited and there is a suppression of B-cell formation of antibodies, limiting the humoral response and resulting in an anti-inflammatory effect (Plumb, 2018; Stafford, 2017). The initial recommended dose begins at 10mg/kg BID with little secondary effects noted at this low dose. The advantages of mycophenolate lean on being cost-effective, rapid efficacy and a PO and IV formula (Stafford, 2017).

Currently, short-term control of CE seems to be achieved when including prednisolone or budesonide (Dandrieux, 2016). Cyclosporine seems to have an important role as adjunctive when refractory to prednisolone alone but long-term control is still questionable as there is reported to have several adverse effects. In a period of six months, a study reports 70% response to treatment, however in three years, only 25% were still responding to the combination of prednisolone and cyclosporin (Allenspach, Rüfenacht, et al., 2006; Allenspach et al., 2007). As so, further studies are needed to assess long-term control effectiveness (Dandrieux et al., 2013).

In conclusion, several randomized controlled trials (RCTs) supports that it is beneficial to induce the treatment with glucocorticoids in IRE/idiopathic IBD dogs. Prednisone alone, budesonide alone (in short-term), or prednisone combined with metronidazole have the same success rate in canine IBD. The evidences of the use of cyclosporin in steroid-refractory cases are inserted on grade III quality of evidence grading guidelines (meaning small descriptive cohort studies), reflecting the need of further studies (Makielski et al., 2018).

1.5.1.4 Prebiotics and Probiotics

While prebiotics have been defined as ‘nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already established in the colon, and thus in effect improve host health’ (Gibson & Roberfroid, 1995), a probiotic is defined as ‘live microbial feed supplement which

beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989). In humans IBD, the usage of probiotics has been showing therapeutic and immune modulating evidences on intestinal inflammation and allergies. Prebiotics had also good results reported as being part of the treatment directed to normalize and restoring an healthy gut microflora (Barko et al., 2018; Gibson et al., 2010).

In 2004, Gibson updated the definition of prebiotics for "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the GI microflora that confer benefits upon host wellbeing and health'. Usually prebiotics are non-digestible, with GI absorption, hydrolysis and gastric acidity resistant fiber compounds such as disaccharides (lactulose, tagatose), oligosaccharides or polysaccharides [fructo-oligosaccharides (FOS), mannan-oligosaccharides (MOS), xylooligosaccharides, polydextrose, galactooligosaccharides] or long-chain prebiotics like inulin, with selective influence on the composition and/or activity of the GI microbiome, while being benefit to the host organism as well (Barko et al., 2018; Gibson et al., 2010). As they pass undigested through the GI tract, they are posteriorly fermented by colonic bacteria resulting in beneficial end-products such as short-chained fatty acids (SCFAs) with important anti-inflammatory properties (modulating the Treg cells) as well as epitheliotropic and essential nutritious capacities. It is also believed that SCFAs influence the mucus layer, increase the number of epithelial cells, elongate the microvilli and avoiding the adherence of pathogenic strains to the epithelial cells. (Barko et al., 2018). A study reported that feeding prebiotics to dogs has apparently no effect on the intake and digestibility of nutrients except for crude protein digestibility that quadratically decreases. However, prebiotics at a dose of 1.40% dry matter intake result in an increase in beneficial bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. with concurrent increase in the SCFA productions which advantageous properties have previously been described (Patra, 2011), demonstrating that using prebiotics as a food supplement may positively influence canine's intestinal health. Further studies are needed in order to evaluate the therapeutical use and clinical efficacy of prebiotics supplements in canine CE.

The most often used probiotics in humans and animals are composed by bacteria such as *Enterococcus*, *Lactobacillus* and *Bifidobacterium* (Sauter et al., 2006) or *Saccharomyces boulardii* (D'Angelo et al., 2018). It is believed that probiotic bacteria have a role in the modulation of the other intestinal microbiota, as well as direct influence on the production of immune-regulating cytokines (Hart et al., 2004; Sartor, 2005; Sauter et al., 2005). Some of the mechanisms observed in probiotics are maintenance of the barrier function by strengthening the epithelial tight junctions function, increment of the mucin production by the goblet cells and defensins by Paneth cells, competition for epithelial colonization sites thus preventing pathogenic bacteria adherence to the intestinal mucosa and lowering the gut permeability. Concerning the immune response modulation, there is an increasing in secretory IgA, on the

production of anti-inflammatory cytokines and an inhibition of proinflammatory cytokines with concurrent promotion of tolerogenic DCs, Tregs and NK activity. They also reduce the pathogen adherence and synthetize antimicrobial peptides and antitoxin immunomodulatory metabolites such as short-chained fatty acids (with enterocyte growth stimulating and anti-inflammatory properties) and secondary bile acids (also with anti-inflammatory properties) as well as bacteriocins and microcins, able of suppressing the other bacteria growth (Gallo, Passaro, Gasbarrini, Landolfi, & Montalto, 2016; Silke Schmitz & Suchodolski, 2016; Suchodolski, 2016).

A study aimed to evaluate if a 21-day probiotic supplementation in addition to an elimination diet based on novel protein sources (salmon and trout), canola meal and rice, composed by three different *Lactobacillus* spp. strains of in FRE dogs could have beneficial effects on intestinal cytokine patterns and on microbiota, hypothesizing that probiotic bacteria may up-regulate the levels of regulatory cytokines in the intestine and balancing the intestinal microbiome. All dogs decreased their CIBDAI level reflecting clinical improvement, duodenal IL-10 mRNA levels decreased while colonic IFN-γ mRNA levels increased, the amount of *Lactobacillus* spp in the feces increased whereas the amount of *Enterobacteriaceae* decreased, which indicates a normalization of the intestinal microbiota after treatment (Sauter et al., 2006). More recently, another study assessed to evaluate the usefulness of using a seven days probiotic supplement composed by *Enterococcus faecium* SF68 with a concurrent standardized high-quality diet and metronidazole combination, in shelter dogs with diarrhea. By day seven, there was a normalization of the stool consistency when compared to those who received metronidazole alone. As so, the addition of SF68 appears to enhance the clinical improvement (Fenimore, Martin, & Lappin, 2017). SF68 has been registered to have beneficial effects on maintaining fecal microbiota, increment of serum and fecal IgA and on the modulation of the immune response (Benyacoub et al., 2003; Fenimore et al., 2017).

Two studies from the same author, both 6 weeks trials, reported that following a hydrolyzed elimination diet + symbiotic probiotic (*Enterococcus faecium* + fructo-oligosaccharides) there was no significant difference between treatments either for inflammation or for intestinal cytokine gene expression when compared to another group that did not receive probiotics. Both studies were likely underpowered due to its small sample size of 12 individuals (Schmitz et al., 2015; Silke Schmitz, Werling, & Allenspach, 2015).

A 8 week study intended to compare the clinical responses to treatment in dogs with canine IBD using a combination therapy (prednisone and metronidazole) or probiotic multi-strains (VSL#3) in dogs with IBD. During the trial, the GI clinical signs resolved as the CIBDAI improved and the histologic index improved in both groups, however exclusively VSL#3 group registered an upregulation expression of select tight junction proteins (TPJ) (Rossi et al., 2014). As so, using VSL#3 may be associated with the exercised protective effects and enhancement

of the mucosal barrier integrity (Dai, Zhao, & Jiang, 2012; Makielski et al., 2018). Also, a correction of the previous dysbiosis was observed, as well as decrease in CD3+ T-cell infiltration in the clinical and histological score enhancing the positive influence of using VSL#3 as an effective probiotic (Rossi et al., 2014). This probiotic has already shown positive effects on preventing, treatment and maintenance of remission in human's ulcerative colitis. Another 8 week trial evaluating the clinical, microbiological and mucosal homeostatic effects of probiotics on the mucosal microbiota in IBD dogs was also performed (White et al., 2017). This study aimed to compare two groups that received a diet + prednisolone treatment, but to one of them probiotics were added. White et al. concluded no differences in treatment response, since both reflected clinical remission, no histopathologic inflammation improvement and an increment in the numbers of total mucosal bacteria. Additionally, as in previous studies, there was an increase in the number of cells expressing select tight junction proteins (TPJ) in the intestinal tissue exclusively in the probiotic-treated group, suggesting that probiotics may be beneficial for mucosal homeostasis and integrity. However, the use of an elimination diet is a common feature of all groups of the two studies, making difficult to evaluate if the beneficial effects were entirely due to the probiotics and not related to the diet (Makielski et al., 2018). Nonetheless, these studies support the idea of being a positive clinical influence on the response to use dietary treatment along with multi-strain probiotics.

Segarra in 2016, compared the results of long-term management in IBD dogs of hydrolyzed protein + an oral supplement composed by chondroitin sulfate and prebiotics (resistant starch, β-glucans and mannaoligosaccharides) with another group that followed the same treatment but without prebiotics in a 6 months trial. There were no differences between groups concerning the clinical score, histopathology and fecal microbiota but the combined administration of the supplement with hydrolyzed diet was safe and registered improvements in selected serum biomarkers, possibly suggesting a reduction in disease activity. This study was then likely underpowered due to its small sample size, highlighting the need of larger studies to confirm the achieved results (Segarra et al., 2016).

In CE dogs to which it was administered *Saccharomyces boulardii,* a non-pathogenic yeast, the clinical activity index, stool frequency, stool consistency and body condition score improved significantly in the CE group versus the placebo, reflecting positive results when in addition to the standard therapy (D'Angelo et al., 2018).

The use of complementary approaches including prebiotics and probiotics has increased in human medicine, especially in IBD cases (Esters & Dignass, 2014). Its use is currently in target of several studies about how the manipulation of the microbiome in dogs can influence the development and progression of chronic enteropathies. Alterations in the fiber or protein percentages, fecal microbiota transplantations and the utilization of bacterial strains in

prebiotics and probiotics are able to induce some changings the overall microbiome, with various impact in the mucosal integrity and in the immune response.

1.5.1.5 Cobalamin (Vitamin B12)

Cobalamin is a water-soluble B complex vitamin – B12. Commercial pet foods have an adequate percentage of dietary cobalamin linked to animal-based dietary protein, one of the reasons why dietary originated deficiencies are uncommon. When in the stomach, the B12-dietary protein molecule contacts with pepsin and HCl and dissociate. Cobalamin then binds to R-protein and it is transported to the small intestine, where R-protein is degraded by pancreatic proteases and the free cobalamin binds to intrinsic factor (secreted by the exocrine pancreas). This new complex formed by intrinsic factor and cobalamin is absorbed by specific receptors in the ileum (Allenspach, 2019; Berghoff, Parnell, Hill, Suchodolski, & Steiner, 2013).

As so, hypocobalaminaemia may either be associated to ileum malabsorption secondary to destruction and/or reduced expression of the cobalamin-IF receptors on ileal enterocytes, likely reflecting distal or diffuse form of small intestinal chronic disease (Allenspach, 2019), indirectly to exocrine pancreatic insufficiency (Batchelor, Noble, Taylor, Cripps, & German, 2007) or dysbiosis, as bacteria compete for cobalamin availability (Giannella, Broitman, & Zamcheck, 1971).

According to the Gastrointestinal Laboratory at Texas A&M, hypocobalamin (<400ng/L) (Steiner, n.d.) is verified to be related to massive distal small intestine (ileum) inflammation and can be a cause of refractoriness to treatment, reflective of a negative outcome (Berghoff et al., 2013; Toresson, Steiner, Suchodolski, & Spillmann, 2016). Low cobalamin levels can also result in villous atrophy and reduced GI function and cell renewal, as B12 as role on the cellular turnover (Hill, 2013). As so, these values should be measured and subcutaneous supplementation should be applied once weekly for 6 weeks, in the following doses of (<15kgbodyweight): 500 µg or (>15kg bodyweight): 1000µg parenteral cobalamin should be reevaluated after treatment and taken as a routine if lowered (Allenspach, 2019; Suchodolski & Allenspach, 2019).

1.5.2 Protein-losing enteropathy (PLE) therapy considerations

As referred before, PLE is universally recognized as a complication of CE although it can also have a primary cause such as primary or secondary lymphangiectasia or intestinal lymphoma (Allenspach et al., 2007; Craven et al., 2004; Dandrieux, 2016; Dossin & Lavoué, 2011; Peterson & Willard, 2003). The treatment of PLE dogs is particularly based on clinical stabilization of the protein-loss and CE clinical signs, starting by normalizing the levels of albumin and achievement or maintenance of an adequate clinical presentation, with a multimodal approach with both dieting and immune-modulation strategies (Gaschen et al.,

2013; Peterson & Willard, 2003). However, if lymphangiectasia is observed, some adaptations should be considered.

1.5.2.1 Diet

Dogs with PLE are often in negative energy balance and reflect specific nutritional needs (Hill, 2013). In the particular case of PLE dogs secondary to lymphangiectasia, and in case of slight clinical signs, these dogs can have a positive response to an hyperdigestible, low-fat (10-15% on a dry-matter basis), highly bioavailable protein diet-changing treatment alone (e.g. Hill's Prescription Diet® i/d® Low Fat GI Restore or Royal Canin® Gastrointestinal™ Low Fat). Besides it is reported the importance of a low percentage in crude fiber, as it can worsen the nutrient absorption (Gaschen et al., 2013; Hill, 2013; Peterson & Willard, 2003). Many clinicians report, when the protein-loss is consequence of CE, good success rate when prescribing an exclusive diet of hydrolyzed proteins or a novel protein diet (Gaschen et al., 2013; Rudinsky et al., 2017; Rudinsky et al., 2018).

A follow up in one to two weeks should be done with the observance of the clinical signs and albumin concentrations (Dandrieux, 2016). If there is no response within this period to the new diet - clinically and by the increasing of the albumin levels – or if there is deterioration of the clinical state, a more aggressive therapeutic approach should be taken in place with simultaneous immunosuppressant drugs and dietary changings, with previous intestinal biopsy (Dandrieux, 2016; Dossin & Lavoué, 2011; Peterson & Willard, 2003). Several studies reports the importance of keeping this new diet as a lifelong treatment, but the efficacy of using the dietary treatment alone is still in need of further investigations (Gaschen et al., 2013; Nakashima et al., 2015; Peterson & Willard, 2003).

The treatment of predisposal breed for PLE - Yorkshire Terriers – revealed benefits when combining a fat restricted diet and immunomodulating medications (Okanishi, Yoshioka, Kagawa, & Watari, 2014). The therapeutic success observed in PLE dogs due to intestinal lymphangiectasia supports the idea of restricting the percentage of fat as beneficial. The argument of using a fat-restricted diet relies on the fact that dietary fat increases the circulating lipids in chymal lymphatic fluid, which increases the lacteal pressure and causes the lymph outflow, aggravating the protein-loss across the intestinal mucosa with worsening of the clinical signs (Dossin & Lavoué, 2011; Simmonds, 1954).

However, a more recent study developed in Yorkshire Terriers - all of them with a clinical score below 12 - reported short-term positive response between one week and one month (as reported before by Allenspach et al., (2007) and Walker et al., (2013)), to the usage of low-fat diets alone without concurrent use of immunosuppressants (Rudinsky et al., 2017). Yet, this study was then again only with Yorkshire Terriers' individuals, which can limit the extrapolation for other PLE breeds.

All things considered and at the current point of investigations, dietary management should be recognized as a first-choice treatment. As so, and bearing in mind its side effects, immuno-modulating approach should be postponed and kept to PLE dogs that are severely clinically unstable or that fail to respond in a short-term period to the diet changing approach.

1.5.2.2 Colloids Fluid Therapy

Albumin is the principal determinant (approximately 80%) on balancing the oncotic pressure, forcing the fluids to remain in the vascular compartment (Chan, 2013). The normal ranges are for dogs 2,6-3,5 g/dL (Mackin, 2016). We characterize as mild hypoalbuminemia values from 2,0 to 2,6 g/dl, moderate hypoalbuminemia from 1,5 to 2,0 g/dl and severe hypoalbuminemia concentrations under 1,5g/dl.

Severe hypoalbuminemia with concurrent chronic diarrhea may suggest PLE (Willard, 2015) and results in lymph drainage, conducing to edematous signs such as cavitary effusions (ascites and pleural effusion) and tissue edema (e.g. subcutaneous, gastrointestinal wall) (Chan, 2013; Mackin, 2016). Dyspnea and tachypnea may warn the owner and the clinician that one of the previous disorders is currently in progress and a thoracic or abdominocentesis may be required. The development of tissue edema predisposes to increased tissue oxygen debt, increasing the risk of bacterial translocation and motility and absorption disorders such as ileus, abdominal discomfort, vomiting and regurgitation.

Fluid therapy with colloids is recommended when the serum albumin concentrations are under 1,5g/dl (Trow, Rozanski, deLaforcade, & Chan, 2008; Viganó, Perissinotto, & Bosco, 2010). Colloid synthetic solutions such as dextrans and hydroxyethyl starch are composed by larger molecules that remain into the vascular space simulating albumin's role on maintaining the oncotic pressure and avoiding extravasation of lymph for the cavities.

A natural colloid formulation composed by human serum albumin (HSA) is also a possibility, though its beneficial effects on PLE dogs is still not determined. As so, and considering its extremely high cost, HSA is saved for emergency situations and critically patients with severe signs of edema. As this is an exogen protein, there is a risk of developing immune reactions, being its repeated administration contraindicated (Gaschen et al., 2013; Neiger, 2013). Two studies were developed evaluating the use of human albumin in critically ill dogs with hypoalbuminemia – one, reported that HSAl significantly increased total protein, serum albumin and oncotic pressure in the survivors, yet, there was a high mortality of the population. The authors couldn't conclude whether it was associated with the administration of HSA or consequence of the underlying disease (Trow et al., 2008). The second study registered as safe the administration of 5% HSA as a constant rate infusion (CRI) at a rate of 2mL/kg/h over 10 hours (daily volume of 20mL/kg/d), increasing the albumin levels to 2,0g/dL in an average of four days. Any of the patients included had experienced major or minor adverse reactions

such as anaphylaxis, angioedema, and urticaria. On the other hand, a recent publication reports that the repeat infusion of HSA for more than 7 days is not recommended based on the observance of non-immune anaphylaxis and delayed (type III) hypersensitivity reactions with concurrent development of anti-HSA antibodies in both healthy and sick dogs (Walker, 2017).

Specific canine-albumin may be promising due to its lower potential for adverse reactions. Its usage is reported to be safe to administer but the accessibility to this product is still restricted and no studies were made concerning its value on CE (Chan, 2013; Craft & Powell, 2012).

Plasma transfusion is excluded as an option due to large volume implied on the increment of the serum albumin concentrations to its normal concentrations – 45 ml/kg of plasma to increase 1g/dl of the host's serum albumin - increasing the risk of volume overload and edematous signs (Hill, 2013).

However, if the primary cause of protein-loss is not corrected, the levels of albumin will repeatedly decrease. Recent studies report that synthetic colloids are rarely effective for more than a day (Mackin, 2016).

Lower levels of albumin can be used as a predictive and early prognosis marker of relapsing. The standardization of the albumin levels should be taken as a significant point of treatment and monitorization of the disease (Dossin & Lavoué, 2011). Ascitis and pleural effusion are clinical signs that are currently associated with PLE, although they're not a reflection of the severity of the prognosis (Simmerson et al., 2014), that is often guarded.

Studies concerning the PLE predisposal breed Yorkshire Terrier, report a long-term response using a combination of diet, prednisolone and metronidazole (Simmerson et al., 2014) but there are also studies that report a long-term positive respond using diet alone (Rudinsky et al., 2017).

1.5.2.3 Antithrombotic Therapy

Dogs with PLE are commonly hypercoagulable and are considered at risk for thromboembolic complications (Hill, 2013) due to an excessive loss of anti-thrombin III (ATIII) which has a similar molecular weight to albumin and α1-PI (Steiner, 2003).

Despite a positive clinical response to treatment and improvement of the clinical state, PLE dogs continued in a hypercoagulable state. As so, they're still predisposed to thromboembolic complications after treatment (Goodwin et al., 2010), reason why the prothrombotic state may be considered multifactorial and not solely related to ATIII's loss . These findings were in agreement with a recent study that reported that antithrombin levels are not correlated with albumin levels and that after 15 days of immunosuppressive treatment all dogs remained in an hypercoagulable state, despite the improvement of the clinical activity and albumin levels,

suggesting that pathogenic factors other than loss of ATIII may influence the hypercoagulability state in PLE dogs (Allenspach, 2019).

The administration of antithrombotic therapy with a low-dose aspirin (0.5–1.0 mg/kg PO SID) or clopidogrel (1–3 mg/kg PO SID) is by now indicated for arterial thromboembolism (Hill, 2013) although thromboprophylaxis efficacy in PLE cases is still a topic in need of further studies.

1.5.3 Innovative therapeutical options

1.5.3.1 Fecal Microbiota Transplantation (FMT) – a promising therapy for IBD?

Modulating the intestinal flora by fecal transplantation, and consequently enhancing the innate immune response, has been showing to be promising in human treatment of GI diseases, especially in Crohn's disease and C*lostridium dificille* recurrent infections (Aroniadis & Brandt, 2013; Kelly et al., 2015). However, in veterinary medicine, its effectiveness is still a field under research.

In 2013, Weese reported that fecal consistency was improved in a 24h period and a normalization of the clinical state for at least 3 months after-treatment in dogs submitted to fecal transplantation (Weese, Costa, & Webb, 2013).

A study was developed in puppies with canine parvovirus infection, comparing the results between a standard treatment and a standard treatment with concurrent fecal microbiota transplantation (FMT). The results were positive, with lower rate of mortality in the FMT group, a faster resolution of the diarrhea and a lower time of hospitalization (Pereira et al., 2018). This study supports the idea that manipulating the microbiota may have a benign impact on changing the course of gastrointestinal diseases.

According to the following FMT protocol, fresh fecal samples are obtained from healthy donors (non-obese adults that produce more than 50g of feces, that do not have history of GI disease, alimentary allergies or atopic diseases, up to date vaccination against important infectious diseases like distemper, parvovirus, adenovirus and leptospirosis, that did not take antibiotics in the past three months before transplantation or with suspicion of bacteria, virus, parasitic or fungi infections and administered by enema to a dog with refractory IBD. Fecal samples are collected before and after FMT and each microbiome is characterized using sequencing of 16s rRNA bacterial genes PCR (Chaitman, 2017; Chaitman et al., 2016).

Rapid-PCR modern assay dysbiosis index (DI) was used for evaluating the impact of fecal microbiota transplantation (FMT) on the microbiota in dogs with chronic enteropathies. In this study, an improvement of dysbiosis was verified at the first week after transplantation, but after three weeks the DI reincreased (Gerbec, Naloga, & Farmacija, 2016). Chaitman delineated in a recent commentary some key aspects of FMT therapy on small animal practice in order to

update the veterinary community about this emergent therapeutical option (Chaitman et al., 2016). The same author also assessed in her study that DI was reported to be significantly decreased in dogs post FMT when compared to dogs before FMT (Chaitman, 2017).

Idiopathic IBD is still a major challenge in veterinary medicine, since it is a common cause of chronic enteritis. Dogs with IBD often follow a combination of therapies, invariably with life-long immunosuppressant medical care due to the risk of relapsing implied, with its secondary effects associated (Dandrieux, 2016). As so, it is important to develop efficient and at the same time safer therapies with lower probability of developing adverse effects. FMT is believed to comply this standard and has been target of several recent researches as therapeutical option, especially in non-responsive CE. At the current point of investigations, the benefits are still short-termed lasting from weeks to a few months.

1.5.3.2 Stem Cells Therapy

Since the gut epithelial surface is constantly in contact and susceptible to permanent aggression from the luminal contents, it is associated with high rates of cell death per day (an average of more than 10^{11} cells daily in humans (Barker, 2013)). This urges the need of a daily turn-over, which is allowed by populations of adult stem cells that are founded within niches that are specialized microenvironments able of providing factors that regulate and support stem cell survival and function (Barker, 2013). This self-renewal capacity hypothesizes the use of these intestine stem cells in for regenerative medicine purposes.

The usage of mesenchymal stem cells (MSCs) has been shown to be effective on humans Crohn's disease due to their immunosuppressant and tissue regenerating competences. In Veterinary Medicine, the interest relies particularly on the refractory cases.

Studies about this emergent therapy for treating inflammatory conditions have been developed (Gattegno-Ho, Argyle, & Argyle, 2012). Pérez-Merino et al., (2015) reports significant positive results in his study with the IV injection of allogeneic adipose tissue-derived mesenchymal stem cells in 9 of 11 IBD dogs, with macroscopically improvement of the GI lesions and a slight reduction of the inflammation, confirmed microscopically.

Recent techniques allowed the in vitro generation of complex tridimensional self-organizing structures derived from in-vitro cultures of stem cells in the absence of a non-epithelial cellular niche, called organoids (Sato et al., 2009). In this case, our interest relies into multipotent intestinal stem cells (ISCs) called enteroids/colonoids - according to its origins, small or large intestine respectively - found near the basis of the intestinal crypts and its differentiation into crypt-villus organoids (Carlone & Breault, 2012; Zachos et al., 2016). Canine intestinal crypt-derived organoids have recently been described (Meneses et al., 2017; Powell & Behnke, 2017). Due to its self-renewal, ability to be expanded in culture while remaining genetically stable and differentiation capacities, the intestinal organoids derived from canine

mesenchymal stem cells may offer regenerative and consequently mucosal healing options in the treatment of CE (Burgener, 2019; Meneses et al., 2016; Mochel et al., 2018; Tesori, Puglisi, Lattanzi, Gasbarrini, & Gasbarrini, 2013).

On the light of the canine intestinal crypt-derived organoids that have recently been described a crypt isolation protocol was established for *in vitro* models of small intestinal and colonic organoids, bringing significant advances and prospects concerning the use of regenerative medicine as an innovative treatment option (Burgener, 2019; Meneses et al., 2017; Powell & Behnke, 2017). Nevertheless, the usage of stem cells is still a very expensive option and requires a lot of specialized equipment for its development.

1.6 PROGNOSIS

Close monitoring consultations should embody the observance of the clinical response to therapy, considering the risk of relapses associated in CE. Measuring the levels of albumin, especially in PLE cases is also important.

Factors of a reserved and poor outcome prognosis are: high CIBDAI, endoscopic observed lesions of the intestinal mucosa, hypocobalaminaemia, hypoalbuminemia, hypovitaminosis D (Jergens et al., 2003; M. Craven et al., 2004; Allenspach et al., 2007; Titmarsh et al., 2015) and CCECAI (Allenspach et al., 2007). In addition, there is evidence that C-reactive protein, serum canine pancreatic lipase immunoreactivity and fecal alpha-1 proteinase inhibitor concentrations are more often increased in PLE cases, reflecting their shortest survival times (Allenspach, 2019).

Clinical activity indexes based on scoring systems such as CIBDAI and CCECAI can also be used for evaluating the prognosis. However, CIBDAI prognostic value is controversial as CCECAI reflects more accurate predictions on the clinical outcome by including serum albumin concentrations and assessment of ascites and pruritus (Allenspach et al., 2007). A CCECAI that's superior to 12 – very severe disease - at the time of diagnosis is significantly correlated to a refractory CE (NRE) resulting in a short-time surviving time, meaning euthanasia in the average time of 3 years (Allenspach et al., 2007). Notwithstanding, more recent studies affirm that the average lifespan is not significantly linked with the attributed scores at the time of diagnosis (Equilino et al., 2015; Gianella et al., 2017) meaning that they should not be used to predict the clinical outcome.

As referred above, the observation of severe lesions in the duodenal mucosa is significantly associated with a negative clinical outcome whereas the severity of the histopathological observing and score is shown to not be correlated with the outcome (Allenspach et al., 2007).

Allenspach et al., (2007) also reinforced the idea that CE dogs with cobalamin levels lower than 200ng/L should be supplemented, as hypocobalaminaemia being a negative prognostic factor associated do the levels of albumin, although the Gastrointestinal Laboratory at Texas A&M reports that levels under 400ng/L should be supplemented (Heilmann & Steiner, 2018; Toresson et al., 2018, 2016). The same Allenspach (2007) study affirms that albumin concentrations under 20g/L were highly related with a negative outcome and strongly associated to refractoriness to treatment. Simmerson et al., (2014) study reinforces this idea by documenting that the clinical outcome and lifespan is directly correlated with the severity of low levels of serum albumin and urea. However Nakashima et al., (2015) affirmed that high levels of urea with the presence of hypoalbuminemia depicting the severity of the low concentration of serum albumin, were negatively correlated with the prognosis. Another study supports the idea that the survival time is not influenced by the level of albumin at the time of diagnosis (Equilino et al., 2015). CE with concurrent protein-loss is considered to have a guarded prognosis due to the uncertainty of the clinical outcome and response to treatment, as well as the higher risk of relapse (Bota et al., 2016).

Serum 25(OH)D concentrations has been shown to be significantly lower at time of diagnosis in dogs with CE, stating the idea of being an acceptable predictive factor for estimating the clinical outcome in dogs with CE. However its role is still in need of further studies (Titmarsh et al., 2015).

Increased serum cPLI is also reported as negatively affecting the outcome (Kathrani et al., 2009).

Secondary findings founded at abdominal ultrasound (e.g. edematous signs as free fluid, pancreas edema, and intestinal wall dilation) can be factors to take into consideration when evaluating the therapeutic success (Jergens & Simpson, 2012).

Relapses are relatively common and the responses to the treatment extremely variable. However, the prognosis is good for mild cases that are responsive to dietary changings. For dogs demanding medical treatment, the prognosis is often guarded to poor. More studies are needed to optimize and verify which treatments are adequate to short and long-term for each type of CE (Dandrieux, 2016).

2 THE LEAKY GUT

2.1 ANATOMICAL AND HISTOLOGICAL FEATURES OF THE MEMBRANE

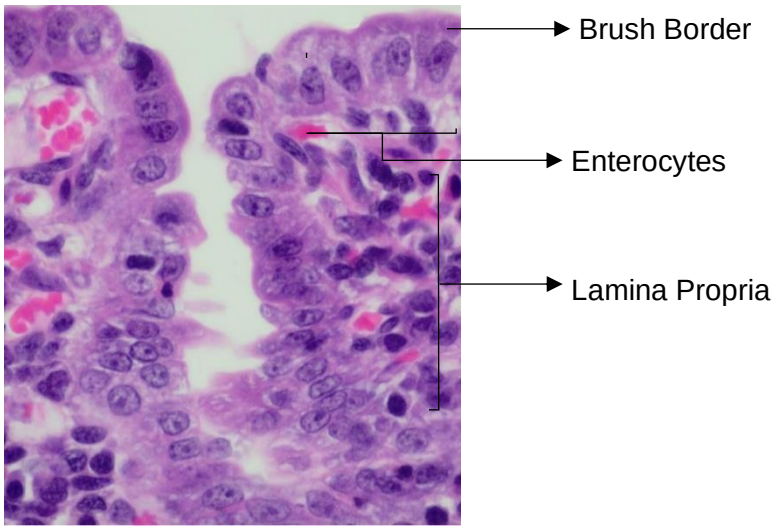
The small intestine extends itself since the distal portion of the pyloric sphincter until the cecum and is comprised by three different portions with the main function of digesting and absorption of nutrients in the ingesta: duodenum, jejunum and ileum. Duodenum is the first portion of the small intestine and the site of entering of the common bile duct which transports bile acids from the gall bladder to the intestine and pancreatic ducts that supplies with pancreatic secretions, at the major duodenal papilla, as well as where the accessory pancreatic duct enters at the minor duodenal papilla, allowing an appropriate digestion of the ingesta. It is located at the right cranial region of the abdomen and connected to jejunum, the longest portion, immediately before the ileum portion. The ileum is then linked to the large intestine at the ileocolic junction in the right caudal region of the abdomen and has the primary function of absorbing fluids, vitamins (such as cobalamin) and electrolytes (Dyce, Sack, & Wensing, 2007).

The ileocecal fold separates the small from the large intestine, and prevents the retrograde flow of the ingesta. The large intestine is the terminal portion of the intestinal tract containing the cecum, colon, rectum and anal canal. It has the main function of absorbing water and nutrients. The cecum is a diverticulum that communicates with the ascending colon by the cecocolic orifice where a mesenteric fold called the ileocecal fold is. The colon, located dorsally in the abdomen, begins cranial and right sided by its ascending portion, and posteriorly passes to the left side cranial – transverse segment - and then progresses caudally along the left side, constituting the descending colon, into the pelvic cavity, where it links to the rectum and the anal canal (Dyce et al., 2007).

The blood supply is mainly supported by the cranial and caudal mesenteric arteries, although there is a part of the duodenum that's nourished by the cranial pancreaticoduodenal branch of the gastroduodenal artery, originated from the celiac artery. The duodenum, jejunum, ileum, cecum, ascending and transverse colon are supplied by the cranial mesenteric artery while the descending colon and the rectum, are supplied from the caudal mesenteric artery, branched from the aorta. The small intestine drains to the liver via portal vein (Evans, 2016). There are several mesenteric lymph nodes lying aside the jejune, ascending, transverse and descending colon. Lymph from the central lacteals in the villi drain to the mesenteric lymphatic vessels to the cisterna chily (Hall & Day, 2017). Although the intestine has its own nerve plexus system, it is also controlled by the autonomic nervous system with sympathetic and parasympathetic fibers that can influence the activity of the enteric nervous system (Reece & Rowe, 2017).

The small intestine is composed by four layers: serosa, muscularis, submucosa and mucosa. The inner layer is called mucosa, responsible for the absorption of the nutrients as well as an extremely important barrier function that will be further approach in detail. The mucosa is protected by enterocytes and goblet cells in permanent contact to the gut lumen, forming an intestinal epithelial layer. Tight junctions between the intestinal epithelial cells are an important component of the physical barrier between the lumen and the lamina propria. The submucosa, composed of connective tissue with blood, lymph vessels and a nerve plexus – submucosal or Meissner's plexus - that controls the secretions of the epithelial cells and the blood flow. This plexus is also composed by nociceptors, which are sensory neurons responsible for the perception of pain. The muscularis with propulsion and mixture of the intestinal content functions, controlled by another nerve plexus – myenteric or Auerbach's plexus -, composed by an outer longitudinal and an inner circular smooth muscular fiber, that by forming macroscopic folds – finger-like processes - increases the surface area of contact with the luminal contents by projecting themselves into the lumen. These folds are covered by villus that together with the crypt cells form the functional unit of the intestine - Figure 2. Severe lesions of the villus cells, whether physical or functional, will have repercussions on the animal status of health by dysregulation of the absorption capacity of nutrients, fluids and electrolytes and mucosal barrier malfunction. The secretive and digestive functions of the enterocytes are accomplished by the production of brush-border digestive proteins and by the capacity of absorbing nutrients and water provided by the ingesta in the luminal content. The outer layer is called serosa, covering the intestine from the outside and in continuous with the mesentery, that involves and supports the intestine within the peritoneum into the abdominal cavity (Reece & Rowe, 2017).

Figure 2 *- Canine duodenal microscopic anatomic features (adapted from Jennings & Premanandan, 2017)*



Relying on multipotent intestinal stem cells (ISCs), the epithelium is able to renew itself. Located near the bottom of the intestinal crypts, these cells are undifferentiated epithelial cells that by cell division are capable of migrating towards the tip of the villus and rapidly differentiating themselves into luminal cell membrane of enterocytes with secretive (goblet and enteroendocrine cells), digestive and absorptive capacities as well as M cells and Peyer's Patches, forming the microvillar membrane (due to the high number of microvilli) also known as brush border, and allowing a constant cell renewal (Burgener, 2019; Date & Sato, 2015).

The large intestine is also four-layered however, the innermost layer mucosa is devoid of villus and microvilli and formed by columnar epithelial parallel cells and numerous crypt base goblet cells able of secreting mucus into the lumen by their mucous glands (Date & Sato, 2015; Meneses et al., 2016).

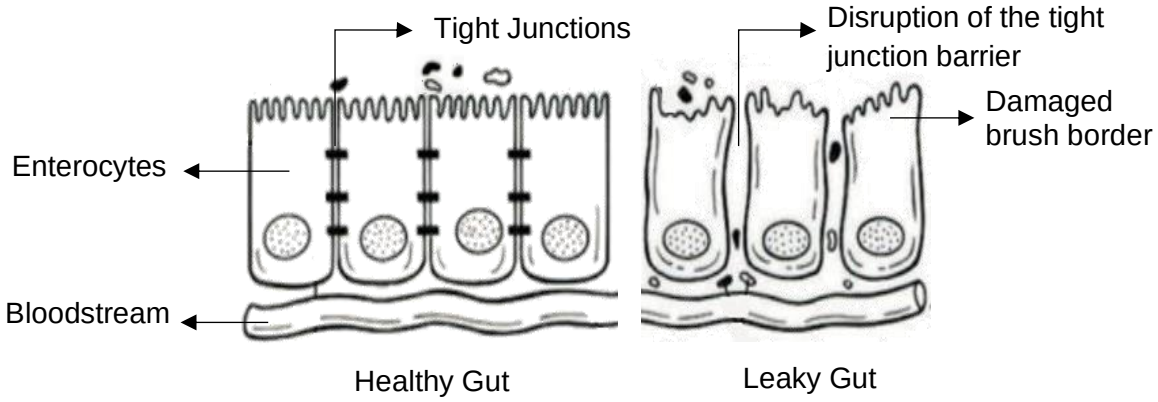
2.2 THE MUCOSAL BARRIER - GUT'S IMMUNOLOGICAL FEATURES

The GI barrier is composed by several anatomic layers, with physiologic and immunologic components. A "leaky gut", meaning a disrupted mucosal barrier, will provide and enhance the development of an immune response and to endorse mucosal inflammation (Ahmad, Sorrell, Batra, Dhawan, & Singh, 2017) . Considering that the intestinal mucosa is in permanent contact with diverse environmental antigens, the barrier must have different mechanisms of approaching and responding to the different antigens: a reaction of tolerance mechanisms to harmless antigens (e.g. commensal bacteria, toxins, environmental and dietary antigens) and the capacity of maintaining the integrity of the membrane, as well as a local reaction on developing an adequate response to invasive pathogens by IgA synthesis, lymphocyte intervention and mastocyte degranulation accompanied by a systemic response with production of several immunoglobulins.

A layer consisting on mucosal epithelial cells joined by tight junctions (TJ) offers the first selective physical effective barrier on limiting the entrance of antigens by promoting apical adjacent cell-to-cell adhesion and blocking the paracellular pathway through the intercellular space, while allowing the passage of water and nutrients (Farquhar, 1963; Luettig, Rosenthal, Barmeyer, & Schulzke, 2015; Powell, 1981). The TJ are mainly formed by claudin family proteins that determine the permeability properties of the epithelium as they can be barrier (claudin-1, -3, -4, -5, -7, and -8) or pore-forming claudins (claudin-2) (Günzel & Yu, 2013). Several studies reported the claudins as mediators of the leaky gut during chronic intestinal inflammation, and therefore, raising awareness about the implication of the TJ on the mucosal barrier since claudin expression alterations – upregulation of claudin-2 - have been reported in humans IBD (Crohn's disease and ulcerative colitis), altering the TJ regular patterns and promoting the leakage (Luettig et al., 2015).

The mucosal layer comprehends an epithelial layer of enterocytes, with tight junctions in between - Figure 3 - covering the lamina propria with a mucosal specialized immune system, the gut-associated lymphoid tissue (GALT). Intestinal absorptive cells uptake the several nutrients from the intestinal lumen while the lamina propria plasma cells produce secretory IgA (Heneghan et al., 2013) contributing for the hosts innate immunity. Goblet cells produce mucins that will coat the gut surface and participating on microbiota sequestration, with high levels of IgA. The epithelial cells also produce several cytokines that will enhance the lymphocyte proliferation by the gut-associated lymphoid tissue and production of IgA and pro-inflammatory components controlling T-cell differentiation (Fukatsu, 2014). Increased mucosal permeability is then also influenced, other than by paracellular flux, by intestinal cell damage (Ahmad et al., 2017).

Figure 3 – *Mucosal epithelial cells and the tight junction barrier (adapted from Kathryn Born's illustration)*

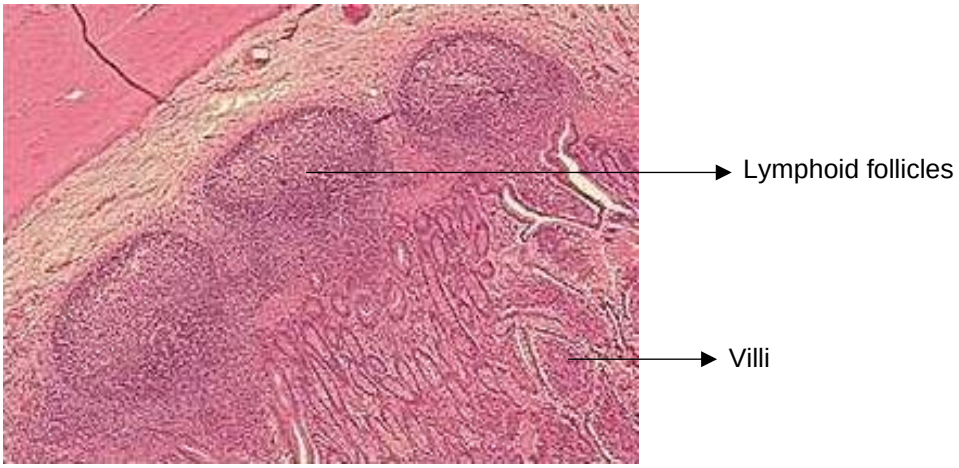


A proper immune response relies on the interaction of the innate and adaptive immune cells, adequately regulated to have a balanced response according to the intensity of the pathogenic infection. The innate (non-specific) immune system is the first line of defense when there is pathogenic invasion. The second set of responses is then established by the adaptive response, when the acquired (specific) immune system becomes activated and produces lasting cells with immune memory capacities, allowing a faster and efficient response once they meet specific pathogens after a first sensitizing encounter (Chaplin, 2010).

2.2.1 Innate Immune Response

The GI mucosa, is a major component of the innate immune system. The mucosa-associated lymphoid tissues (MALTs) has all the types of cells that are needed to initiate an immune response: Dendritic cells, messengers between the innate and adaptive immune systems and T cells and B cells, both components of the adaptive system. The Gut-associated lymphoid tissue (GALT) is a component of the mucosa-associated lymphoid tissue, that lies in the interior of the lamina propria and represents the largest immunological organ exposed to extern antigens (Chehade & Mayer, 2005). It is characterized by disseminated lymphocytes and aggregated follicles of lymphocytes such as mesenteric lymph nodes and Peyer's patches (PP), located in canine species along the jejunum and upper and terminal ileum – Figure 4 (Haley, 2017). These lymphoid tissues, in opposition to lymph nodes, do not to expose the antigen through afferent lymph, directly reacting to the antigens from the surface (Tizard, 2013).

Figure 4- *Canine ileum, showing three lymphoid follicles of a Peyer's patch (image provided by Richard Bowen)*



In the GI tract, this first line of defense comprises the intestinal epithelium with the tight junctions between the enterocytes, the mucin produced by the goblet cells and the innate cells that lie in the lamina propria (dendritic cells, neutrophils, monocytes/macrophages and innate lymphoid cells) (Wallace, 2014). The GALT, attends as antigen sampling and inductive site of the mucosal immune system. The antigens are processed in the inductive sites such as PPs, and the cellular and humoral response developed in the effector sites. There are two possible mechanisms about how protein and peptide antigens can infiltrate an intact intestinal wall into the intestinal lymphoid tissue: by endocytosing in to specialized epithelial cells (M cells) that are directly in contact with the Peyer's patches that posteriorly transport them into the underlying tissue with dendritic cells and macrophages or by directly invading the cytosol of the Dendritic cells (DCs) - which processes cross between the enterocytes contacting to the

gut lumen - and be consequentially presented to T cells in the GALT. Both M and dendritic cells work as antigen-processing cells (APCs).

To be able to play a balanced interaction between the mucosa and the bacteria, and an appropriate identification of what is pathogenic and non-pathogenic the epithelium uses pattern recognition receptors (PRR) that are capable of recognizing structural elements of bacteria. The enterocyte PRRs include a variety of Toll-like receptors (TLR's) and nucleotide oligomerization domain-like receptors (NLRs), that are two innate immunity sensors expressed whether on the surface membrane, in intracellular vesicles or freely on the cytoplasmic compartments of the epithelial cell and which activation leads to the recruitment of innate immune cells (Fukata, Vamadevan, & Abreu, 2009). Toll-like receptors are pattern-recognition receptors that by recognizing the microbe associated molecular patterns (MAMPs) identify the physiological intestinal microbiota, maintaining the gut flora homeostasis. However, and since they are components of the innate immune response, the *"non self"* recognition of a pathogen-associated molecular patterns (PAMPs) specifies the invasion potentially pathogenic organisms (Kathrani et al., 2014), activating innate pathways. Taking macrophages as an example, they don't targeted for specific pathogens but for general features such as LPS of the gram-negative or lipoteichoic acid from the gram-positive bacteria using tool-like receptors (TLR) (Tizard, 2013).

Tool-like receptors, located in the cytoplasmic cell membrane or within intracellular vesicles play, once activated, an essential role on: 1) signaling invasive pathogenic organisms by prompting the releasing of cytokines and 2) favoring phagocytosis of the infected cells. TLR-2 and TLR-4 are capable of recognizing lipopeptides and lipopolysaccharides (LPS), components found in gram-positive and negative bacteria respectively, TLR-5 of recognizing flagellin, a component of the cell body of flagellated bacteria and TLR-9 radars the presence of intracellular bacteria. The nucleotide-binding oligomerization domain 2 (NOD2), located within the cytoplasmic compartments of the epithelial cell, also recognize invasive intra-cellular bacterial LPS and the activates factor nuclear kappa B (NF-kB) pathway, increasing the proinflammatory cytokine and defensins production (Caruso, Warner, Inohara, & Núñez, 2014; Tizard, 2013). As so, NRLs such as NOD1 and NOD2 can act as a secondary defense line when TLRs fail on preventing the invasion of pathogenic identities, and leads, in co-stimulation with TLRs stimulus to the increment of Th1, Th2 and Th17 cell immune responses (Fritz et al., 2007; Magalhaes et al., 2008).

The critical role of TLRs is to function has a first-line barrier to microbial invaders, as so, if they're incompetent, the animal may reflect an augmented vulnerability to infection and lower capacity of defending against bacterial invasion. Additionally, the cell activation through TLR4, TLR5 and TLR9 has shown to be induce defensine molecules production (Palazzo et al.,

2007). In dogs with CE, several polymorphisms in TLRs were observed and consequently, a disrupted immune response can be triggered.

TLR's are upregulated in most of the inflammatory disorders, considering that TLR signaling is associated to the pathogenesis of chronic inflammation (Fukata et al., 2009). The absence of the decrease of the upregulated TLR expression after treatment and clinical significant improvement may bear out the hypothesis that the observation of the over expression of TLRs – TLR2, TLR4 and TLR9 – may be linked to a genetic predisposition to IBD, and as so, can help on predicting the development of chronic enteropathies (Burgener et al., 2008).

A study measured the TLR2 and TLR4 mRNA expression in duodenal biopsies of IBD and healthy dogs, observing in the IBD dogs that the TLR2 mRNA was significantly upregulated in comparison to the control group. The same study also registered mild correlation with the clinical severity of the disease, but no histological correlation associated (McMahon et al., 2010). Lastly, the overexpression of TLR2 mRNA with a concurrent defect innate immune response can enhance the development of canine IBD.

In other study developed only in German Shepherds with CE (FRE and ARE subcategorizations) showed several single-nucleotide polymorphisms in TLR4 and TLR5 genes, reporting a significant upregulation of TLR4 mRNA duodenal, ileum and colonic expression and a downregulation of TLR5 (Allenspach et al., 2010; Kathrani et al., 2010). However, TLR2 and TLR9 expression had no significant difference when compared to healthy dogs (Allenspach et al., 2010).

Two studies verified that two single nucleotide polymorphisms – C100T and T1844C – implied on the codification of TLR5 are confirmed to be CE's protective factors in several included breeds. As so, targeting TLR5 may be helpful on defining new diagnosis and therapeutics, defining new methods of fighting the disease (Kathrani et al., 2011; Kathrani et al., 2010). The same author also confirmed that two SNPs – A1571T and G1807A – in TLR4 play an active role in IBD pathogenesis in German Shepherds as well as the heterozygotism for four SNPs in NOD2 (Kathrani et al., 2014).

DCs are the main connector cells for the initiation of cellular and humoral adaptive immune response (Steinman & Hemmi, 2006) since they're capable of identifying pathogens, phagocytose them and to present its peptides in the major histocompatibility complex (MHC) to T cells and to retain the antigens in an intact form for transporting from the site of infection to the lymphoid organs and presentation to the B-cells (Bergtold, Desai, Gavhane, & Clynes, 2005). The M cells, rather than degrading the antigens, phagocyte and transport them to the underlying lymphoid tissue (PPs) to the effector cells and permit its passage along the intercellular space to the tissue fluid, allowing it to be carried to the draining lymph into the

thoracic duct and onto the blood stream, reentering the lamina propria. An adaptive immune response is then triggered in the local effector tissues.

2.2.2 Adaptive Immune Response

In contrast to the innate immune response, the adaptive immune system is directed towards specific target antigens. An adaptive immune response is then triggered in the local effector tissues, composed by antigen-specific mucosal effector cells, resulting in two forms of immunity called cell mediated immunity, dependent of the activity of the T lymphocytes and a humoral immunity, dependent of the function of B lymphocytes (Chaplin, 2010).

2.2.2.1 Cell Mediated Immunity

The antigen presenting cells mediates the differentiation of the Naïve T cells into effector T helper cells, including Th1, Th2 and Th17 (Wallace, 2014).

Naïve T cells leave the bloodstream into the lymphoid tissue and differentiate into helper T-cells or cytotoxic T-cells, depending on t-cell receptors (TCR) and either CD4 or CD8 receptors respectively, found in T lymphocytes, to bind to a specific antigen fragment (e.g. peptides) of the pathogen. Once expressed the receptor molecules of the major histocompatibility complex (MHC) class I and II at naïve, it is possible for the antigenic peptides to bind and be presented either to CD4+ or CD8+ T-cells, triggering its functions (Steinman et al., 2003). CD4 and CD8 are receptors in the T cell membrane, that are activated once bonded to either MHCI or MHCII. Class I MHC molecules are found on all nucleated cells, and their function is to present endogenous antigens to CD8+ T cells, that become cytotoxic T-cells – T-cell mediated toxicity. Class II MHC molecules are restricted to APC cells (macrophages, DCs and B cells), mast cells and neutrophils, able of presenting exogenous antigens to CD4+ T cells that can posteriorly differentiate into several T-helper lymphocytes – T cell mediated help. In dogs, CD4 is expressed only on neutrophils and macrophages (Tizard, 2013). Helper T-cells are implied on antibody production once they have an active role on activating the B-cells and enhancing phagocytosis by attracting macrophages (Janeway, Travers, Walport, & Shlomchik, 2001a). Once these T cells are activated, they massively replicate and secrete regulatory cytokines, with a role on an active immune response that will be further described. On the other hand, cytotoxic T-cells induce apoptosis by forming transmembrane pores named perforins, thereby allowing the cytotoxic molecules (granzymes) to enter the intracellular compartment (Janeway et al., 2001a).

The uptake of immunocomplexes and the stimulation of the activating and inhibitory pathways in the cell's receptors will determine whether there is DCs activation or not, regulating its tolerance , together with the previous referred function of triggering, phagocytosing and presenting the antigens peptides to the T cells (Nimmerjahn & Ravetch, 2007; Steinman et al., 2003).

There are three major subpopulations of helper T cells: Th1, Th2, Th17. The cytokines by them produced mediated the several immune responses and influence each other's functioning. Th1 cells are stimulated by interleukin-12 (IL-12) and secret IL-2, interferon-γ (IFN- γ), tumor necrosis factor alfa and beta (TNF-α and TNF- β) promoting mostly cell-mediated responses. IL-2 is able to promote Th1 activation and B cell proliferation, to enhance cytotoxicity by increment of the NK cells, to activate macrophages, stimulate hematopoietic cell proliferation and to amplify IFN- γ production. IFN- γ inhibits Th2 cells, promotes and amplifies the Th1 cell activity and activates NK cells and macrophages. TNF-α promotes the adhesion of the phagocytes to the endothelial cells, by changing their permeability. As Th1 cells also recognize antigens presented by lymphocytes B of already activated cells, they play an active role in opsonization, promoting cell differentiation into plasma-cells and consequently enhancing the production of IgG. Th2 cells secrete IL-4, IL-5, IL-10 and IL-13, often promoting antibody responses by stimulating the production of immunoglobulins, mainly IgM, IgA and IgE. IL-10 regulates Th1 and Th17 cell activity by suppressing the activation of Th1 and Th17 cell functioning. Th17 cells are stimulated by IL-6, transforming growth factor beta (TGF-β) and IL-23 and secrete IL-17 are potent B-cell helpers promote neutrophil-mediated inflammation. Indirectly, they also enhance the production of hematopoietic factors, stimulating the granulopoieses in the bone marrow with increment of the number of granulocytes and macrophages. There are also Treg cells**,** resultant of the differentiation of T helper cells with CD4 and CD25 receptors, that secrete suppressive cytokines (like IL-10 and TGF-β) capable of suppressing the activity of other antigenic-specific T-cells. By suppressing the response of helper T cells to antigens, they prevent inappropriate T cell activation in the absence of an antigen, as well as the and macrophage activity and regulate the immune system by maintaining the homeostasis between tolerance and immunity.

Although the GALT has the function to stimulate protective immune responses to pathogens, it should also remain tolerant to harmless antigens such as commensal bacteria and food. The effectiveness of the mucosal barrier is dependent of a combination of factors such as the balance between the mechanisms of sensibility and tolerance to the antigens, the efficiency of the cells of innate and adaptive immune response on the elimination of invasive molecules and the host regulation of the immune response. These mechanisms of tolerance are managed by an arrange of specified cells in GALT, such as regulatory T cells (Treg) that play an important role in cell-mediated mucosal immunotolerance (Junginger, Schwittlick, Lemensieck, Nolte, & Hewicker-Trautwein, 2012; Maeda, Ohno, Fujiwara-Igarashi, Uchida, & Tsujimoto, 2016).

The antigen-presenting cells (APC's) are able to induce mediating cells called antigen-specific regulatory T cells (Tregs) that positively influence the oral tolerance which is the process of inducing a state of a local and systemic non-responsiveness to antigens that access to the organism by the enteric route. In other words, an active process of inducing immunological

tolerance (Commins, 2015; Male, Brostoff, Roth, & Roitt, 2013; Paul, 2013; Tizard, 2013). The establishment of the oral tolerance is crucial on preventing intestinal disorders, as inflammatory bowel disease. Therefore, a depletion of the amount of Treg cells results in loss of tolerance allowing the development of autoimmune and immune-mediated diseases (Nimmerjahn & Ravetch, 2008).

Studies point that the microbiome actively plays an active role on amplifying and maintaining Treg activity. Also, Treg were reported as decreased in duodenal samples of CE dogs (Junginger et al., 2012; Maeda et al., 2016) as in peripheral blood samples of IRE and PLE dogs (Volkmann et al., 2014) leading to the hypothesize the clinical utility of Treg being a useful cellular biomarker on monitoring disease progression and response to treatment. Also, glutamine is the source of energy for enterocytes, justifying the weakening of the epithelial barrier and the lowered immune function efficiency when there is food deprivation or long parenteral nutrition (Newsholme & Carrie, 1994). The gut immunity has been stated to quickly recovers with refeeding, as the amount of enteral nutrition is reported to be correlated to the size of GALT and the secretory IgA levels (Fukatsu, 2014).

In result, cytokines determine T cell differentiation of Th1, Th2, Th17 and Treg cells, resulting in different immune-response pathways. Therefore, cytokines adjust the development, relapse and/or exacerbation of the inflammatory processes. While in human medicine the Crohn's disease is leaning to Th1 cell mediated response and ulcerative colitis to Th2 (Sanchez-Muñoz, Dominguez-Lopez, & Yamamoto-Furusho, 2008), in veterinary medicine there is no consensus between studies that point to either one or another immune-response in CE dogs. This question results from the high variability of cytokines expressed in dogs with CE (Jergens et al., 2009).

2.2.2.2 Humoral Immunity

B-cells are formed from hematopoietic stem cells in the bone marrow and migrate into secondary lymphoid tissues such as lymph nodes, Peyer's patches, and spleen. These cells become activated when its receptor recognizes a specific antigen and subsequently mature into plasma cells able of synthetizing immunoglobulins (Tizard, 2013).

Immunoglobulins, which also constitutes the antibodies, are capable of complexing with specific pathogens by binding to their specific antigenic fragments, expressed extracellularly on infected cells or in peripheral circulation. Once this link is established, the antibody will trigger mechanisms of elimination of either the pathogen or the infected cell. Some of these cells become memory B-cells in circulation, promptly alarming and responding the organism for the presence of a previous insult while the others become antibody-producing plasma cells, capable of synthetizing high titers of antibodies (Chaplin, 2010; Tizard, 2013).

a) Structure, synthesis and circulation of the immunoglobulins

Immunoglobulins are highly specific symmetrically structured of glycoprotein molecules synthetized by B plasma cells, formed by two identical heavy (H) chains bonded to two identical light (L) chains by a combination of non-covalent and covalent interchain disulfide bonds, composing the total of four polypeptide chains. It is also possible to distinguish two regions, one constant (C) region, and a second region, reflecting the amino terminal ends of the polypeptide chains with considerable variation of the amino acid composition, forming the called variable (V) region. Each L chain consists of one constant and one variable domain. The H chain consists on a variable domain and three or four constant domains (CH1, CH2, CH3) according to the Ig class. These glycoproteins can be composed by one or more units, each one with the description above. Each Ig monomer comprises two antigen-binding sites (bivalent Ig), located in the variable regions of the heavy and light chains. The antigen molecule (epitope) if recognized by the antigen-binding site (paratope), will then associate to the immunoglobulin composing an extremely specific interaction (Schroeder & Cavacini, 2010). The interconnection of the antibodies with T-cells and innate immune effector cells guarantees an adequate innate and humoral response, conferring to the host a healthy immune system (Nimmerjahn & Ravetch, 2007).

Immunoglobulins can function as lymphocyte B recognition structures, providing specific stimulation of the different B cell antigen receptors (BCRs) or being secreted by antibody-secreting differentiated plasma cells, resulting into several unique classes of immunoglobulins called antibodies. There are five antibody classes according to the type of heavy chain – IgA(α), IgG(γ), IgM(μ), IgD(δ) and IgE(ε)– allowing them to function according to the type of the immune response required, all originate as B cell antigen receptors. They vary in molecular weight, heavy chain type, serum percentage and concentrations, distribution on the organism and most importantly, function. (Alberts et al., 2002). There is also variance within each class, forming subclasses. In dogs, IgG has the longest serum half-life among the different immunoglobulins composing an average of 85% of the total serum immunoglobulins (1000-2000mg/dl), followed by IgM (70-270mg/dl), IgA (20-150mg/dl) and in minor quantity IgE (2.3-4.2mg/dl) and IgD (Tizard, 2013). Briefly, and concerning the functions of the different classes, IgG is mainly responsible for systemic immune response, while IgM is mostly produced during a primary immune response. IgA is particularly important for the mucosal immunity, exercising a first-line defense on excluding pathogens. While IgE is associated with parasitical infections or allergenic reactions, IgD is found on the membrane of immature lymphocytes and its function is still subject of study. IgE is found in minor quantities, such as IgD.

The immunoglobulins have two main functions, them being the recognition of antigenic molecules by their antigen-binding sites in the variable regions and an immune mechanism effector function, expressed by their constant region. Cell's receptors for immunoglobulins

(FcRs) allow the antibodies to bind by their Fc portion, representing the interface between the effector cells of the innate immune effector cells (mast cells, neutrophils, monocytes and macrophages) and adaptive the immune system, regulating and executing antibody-mediated responses. The same effector cell has inhibitory and activating FcR, resulting in different pathways according to the immunoglobulin specific stimulus, actively participating in the mediation of the immune response. Secondly, they are also modulators of the adaptive immune response by regulating B cell and dendritic cells activation (Nimmerjahn & Ravetch, 2007).

The binding of the immunocomplexes to the different activating and inhibitory FcRs with consequent triggering of activating and inhibitory pathways, sets the magnitude of the cell responses. All of FcyRs types – and therefore dependent of the y chain - express activating pathways, except FcyRIIB that expresses inhibitory pathway. In innate immune effector cells results in cell degranulation, phagocytosis, antibody-dependent cellular cytotoxicity and antigenic presentation, therefore FcyRIIB reflects a regulatory mechanism on preventing nonspecific activation of the effector cells immune response. On B cells, that do not express activating FcRs, FcyRIIB downregulates the activating pathways triggered by signalizing of the B-cell receptor (BCR), that would in its absence synthetize more autoantibodies (Bolland & Ravetch, 1999; Ravetch, 2000). Other than FcyRs, there are other receptors expressed in basophils, mast cells and monocytes for other immunoglobulins isotypes such as FcαRI (Ravetch & Paul, 2003).

A study also found significant correlation between the albumin concentrations and the IgG levels, but not IgM or IgA in both saliva and tears, suggesting that IgG and albumin pass into secretions by passive diffusion. In contrast, IgA and IgM result of an active secretion by the plasma cells located on the GALT (German, Hall, & Day, 1998). The loss of inhibitory FcR is related to loss of tolerance in the humoral response, and the triggering of an autoimmune reaction (Nimmerjahn & Ravetch, 2007).

Although most plasma cells synthetized by the lamina propria lymphocytes in the PP are IgA-producing plasma cells (IgA+) (Pabst, 1987), there is also production of many IgG. In the present study, we relay our interest in immunoglobulins A and G, that will further be approach in detail.

i. Serum and secretory IgA

Immunoglobulins A are characterized by their α-type heavy chain and can be found in two forms: secretory IgA at the mucosal surfaces and serum IgA in much lesser amount into the circulatory system (Macpherson, Hunziker, McCoy, & Lamarre, 2001), both originate from the plasma cells of the GALT by a changement of class derived from IgM. The majority of the antibodies that are expressed at the surface of the mucosa consists on salivary IgA, having a primary role in the primary mucosal tissue immune defenses (Haley, 2017), while serum IgA is part of the systemic immune response (Schroeder & Cavacini, 2010).

There are two subclasses of IgA, that differ mainly in their hinge region. IgA_1 is composed by a longer hinge region with a duplicated stretch of amino acids, resulting in an increased sensitivity to bacterial proteases whereas IgA_2 has a shorter hinge region, granting a lower vulnerability to protease digestion. This finding is explanatory of why mucosal secretions is mainly represented by IgA_2, and serum IgA is almost totally composed by IgA_1 (Schroeder & Cavacini, 2010).

Fc receptors being transmembrane glycoproteins, intermediate the connection between humoral and cellular immune compartment, by allowing the immunoglobulins to bond to the effector cell surface and communicate to the intracellular mechanisms. IgA receptor FcαRI (CD89) is expressed in granulocytes and monocyte, macrophages, and DCs and its signaling can occur through γ-chain that comprises an immunoreceptor tyrosine-based activation motif or not. When cross-linking of FcαRI to an associated γ-chain occurs, FcRs are signaled and trigger a cascade of cytokines liberation and induces effector functions (i.e. phagocytosis and ADCC). Independent of the γ-chain and therefore not implying immunoreceptor tyrosine-based activation motif, it implies the endocytose of the FcRs into endosomes and the posteriorly reprocess of IgA back into the cell surface. FcαRI (CD89) seems to bind to secretory IgA with higher affinity than to serum IgA (Schroeder & Cavacini, 2010).

A study has measured the amount of secretory and serum IgA in seven healthy dogs for three days, in order to assess an average value of the amount of these immunoglobulins. The serum IgA showed much lower values, ranged from 0.27 to 0.31 g/L without variability in the different days of sampling in comparison to ileum secretory IgA, that reflected some variability in the three days of sampling, varying from 3.0 to 4.4 mg/g. The same study also confirms that postprandial measurements do not impact the IgA concentrations in the serum, saliva or ileal content (Flickinger, Grieshop, Merchen, & Fahey, 2004).

A more recent study assessed that serum IgA values vary widely between different breeds (from 0.01 to 3.0 g/L), justifying why the normal range of immunoglobulins is not well established (Olsson et al., 2014).There is no variability according to sex-predisposition (Griot-Wenk et al., 1999; Olsson et al., 2014). However, the production of IgA can be age correlated

as younger dogs have lower values, stabilizing at one to two years old (HogenEsch, Thompson, Dunham, Ceddia, & Hayek, 2004).

The most prevalent canine IgA type is secretory IgA (Ginel, Novales, Lozano, Molleda, & Lopez, 1993; Goldblum, 1990; Mestecky, Russell, & Elson, 1999; Snoeck, Peters, & Cox, 2006). It is formed by two glycoprotein molecules united by a J-chain, composing the immunoglobulin A dimeric (polymeric) form. They are the predominant secretory antibody of the mucosal surfaces (e.g. oral mucosa, biliary, respiratory, intestinal and urogenital tract) and secretions (e.g. bile, saliva, colostrum and milk) as they play a principal role on maintaining the first-line immune defense against enteric antigens, with local anti-inflammatory effects and immune-regulating capacities by preventing bacteria adherence and neutralizing antigens locally (Goldblum, 1990; Macpherson, McCoy, Johansen, & Brandtzaeg, 2008; Rinkinen, Teppo, Harmoinen, & Westermarck, 2003). Secretory IgA - is the primary immunoglobulin involved in protecting mucosal surfaces and is locally produced in the effector tissues by the mucosal lymphocytes and released locally onto the mucosal surface. Its properties, hereafter described, allow them to be proteolysis resistant being able to resist in the luminal surface of the gut and exercise their first-line immune defense barrier function, avoiding the adhesion and invasion of pathogenic agents (Male et al., 2013; Tizard, 2013).

The mucosal cells present on their membrane surface a specific receptor (FcR) named polymeric Ig receptor (pIgR) allows the polymeric IgA to be selective transported across the secretory epithelium - by exocytosis - into the mucosal secretions. A fragment of pIgR will become part of the secretory IgA at the moment they're secreted into the lumen, according to a specific and integrated process to the locals of potential inflammation or pathogenic invasion, granting them the property of resisting the proteolysis (Goldblum, 1990; Snoeck et al., 2006).

Its anti-inflammatory activity is comprised by its capacity of binding to an antigen in the mucosal surfaces, inhibiting its absorption and the triggering of secondary immune-responses such as the production of IgG and IgE. Another functional mechanisms are the circulation of the antigenic-polymeric IgA complexes into the bloodstream to the hepatobiliary system allowing the elimination of these immune complexes from the serum by phagocytose, circulation into the vascular system or excreted into the mucosal lumen for surface defense (Snoeck et al., 2006). Additionally, the polymeric IgA also inhibits the inflow of polymorphonuclears into the inflammatory sites and in contrast to serum IgA, secretory IgA seems not to have a major impression on triggering phagocytosis mechanism complement activation. The activation of the complement in the mucosa would induce local inflammatory reactions that would increase the permeability of the mucosal membrane, thus secretory IgA have in this case an anti-inflammatory capacity, important for maintain the integrity of the mucosal surface by allowing an intracellular destruction of the antigenic-antibody complexes in endocytic vesicles (Goldblum, 1990; Macpherson et al., 2008; Snoeck et al., 2006).

The serum IgA is found intravascularly and are predominantly monomers. Though the role of secretory IgA is well known, the role of circulatory IgA is not fully understood in the canine species, however it is believed to act as a minor mechanism of systemic immunity functioning as a second line defense (Otten & van Egmond, 2004; Rinkinen et al., 2003). Serum IgA is the second most prevalent antibody in serum after IgG, however, its metabolization occurs five times faster (Woof & Kerr, 2006).

The binding of the antibody-antigen complex by its Fc fragment to monocytes or granulocytes cells, initiate several pro-inflammatory immune reactions such as eosinophil and basophil degranulation, phagocytosis by macrophagic cells and monocytes, antibody-dependent cell-mediated cytotoxicity (ADCC) and the activation of the alternative pathway of the complement system. (Monteiro & van de Winkel, 2003; van Egmond et al., 2001). The alternative pathway is a component of the innate immune response, triggered when a microbial cell is recognized by complement plasmatic proteins such as C3 in the blood vessels, synthetized by hepatocytes ad macrophages. When activated, its end-products especially C3b, binds to the cell surface of the invasive pathogens opsonizing them for destruction, activate inflammatory cells and endorses the antibody production by liberating onto the bloodstream antibody-antigen immunocomplexes. ADCC is a slower and less efficient mechanism of T cell-mediated cytotoxicity, allowing the natural killer (NK) lymphocytes or macrophages with FcyRIII to bind with a specific antibody-antigen complex, inducing the destruction of the antigen by releasing of cytolytic granules (Tizard, 2013).

Several studies that compared serum and secretory IgA in dogs documented a poor correlation between the two, reflecting that it is not correct to local IgA relying on the systemic concentrations and vice-versa. However, as decreased concentrations of systemic IgA don't reflect low concentrations of local IgA levels, it is believed that the presence of normal secretory levels may be responsible for an effective immune response against local pathogens or that the organism compensates by rising the production of IgM and the maintenance of a healthy clinical presentation (German et al., 1998; Ginel et al., 1993; HogenEsch et al., 2004; Macpherson et al., 2008; Norris & Gershwin, 2003; Rinkinen et al., 2003).

i.a. IgA deficiency in particular dog breeds

In the human species, IgA deficiency is well characterized by a low to absent serum IgA (<0.07g/L) associated with normal to elevated serum concentrations of immunoglobulin G (IgG) and immunoglobulin M (IgM) (Batt, Barnes, Rutgers, & Carter, 1991; Norris & Gershwin, 2003). In both humans and dogs, IgA deficiency is associated with recurrent infections in the mucosal sites and with allergy, auto-immune and immune-mediated diseases and as part of the pathogenesis of the disease conduction (Brandtzaeg, 2010; Olsson et al., 2014).

Due to the high variability of the register serum measurements of IgA in the different breeds, it is difficult to assess a cut-off that defines IgA deficiency. Significant low serum and particularly low secretory IgA concentrations are commonly found in clinically healthy German Shepherds and Shar-Pei dogs in comparison to other breeds (Batt et al., 1991; Olsson et al., 2014; Rivas et al., 1995). However, Olsson et.al also reported very low levels of serum IgA (using the human cut-off of <0.07g/l in comparison to the normal average levels of 0.27-0.31 g/L) in Hovawart, Norwegian elkhound, Nova Scotia duck tolling retriever, Bullterrier, Golden retriever and Labrador retriever.

As it has been confirmed that the number of IgA producing plasma cells it is not significantly altered, it is thought that the production of the IgA in the German Shepherds GALT is somehow lowered (German, Hall, & Day, 2000; Littler, Batt, & Lloyd, 2006; Peters, Calvert, Hall, & Day, 2004), suggesting a deficient synthesis or secretion of the immunoglobulins to the mucosal surface (Batt et al., 1991; Ginel et al., 1993).

In conclusion and bearing in mind the importance of the serum IgA on maintaining an adequate mucosal immune response, it is believed that dogs with IgA deficiency are predisposed for developing bacterial overgrowth and chronic enteropathies, supporting the hypothesis that the local immunity might be compromised as the presence of antigens is supposed to stimulate and increase the levels of secretory IgA. IgA deficiency is confirmed to be associated with an increased susceptibility to enteric infections, predisposing the dogs affected to inflammatory bowel disease and bacterial overgrowth (Batt et al., 1991; German et al., 1998). The measurement of secretory IgA of the duodenal juice, may provide important information about their immunological status with prognostic and therapeutic interest, though subnormal levels of IgA may be present in clinically healthy dogs.

i.b. IgA in CE dogs

Since the most common infiltration cell type of CE is lymphocytic and plasmocytic, it would be expected to verify an increment of the amount of T cells, particularly T-helper cells (CD4+), and IgA+ plasma cells in the small intestine. A study developed in 2013 aimed to assess if the IgA expression is altered in dogs with IBD, concluding that IBD dogs show significantly decreased fecal and duodenal (secretory) IgA concentrations and fewer IgA+ peripheral blood mononuclear cells (PBMCs) in comparison to healthy dogs (and dogs with lymphoma) as well as significantly lower lamina propria IgA+ cells in the duodenal mucosa, which predisposes for the development of chronic enteritis (Maeda et al., 2013). However, there was no difference observed between the healthy and IBD group, concerning the levels of serum IgA. This is consistent with what has been found in previous reports.

In immunoproliferative enteropathy of the Basenjis, the mucosa seems to be infiltrated by lymphocytes, plasma cells and some neutrophils with a concomitant polyclonal increase in

serum IgA. Gluten-sensitive enteropathy of Irish Setters, also shows mucosal infiltration of lymphocytes and other inflammatory cells, with an increased number of CD4+ plasma cells and elevated serum IgA levels (Tizard, 2013).

ii. Serum IgG

Immunoglobulins G – characterized by their γ-type chain - is the most abundant (an average of 85% of the total) immunoglobulin found intra and extravascular comprised in four IgG subclasses: IgG_1, IgG_2, IgG_3 and IgG_4 and is produced as part of the systemic defense to an insult (Flickinger et al., 2004). The different IgG subclasses, exercise different functional proposes.

IgG act by immune elimination, while IgA act predominantly by immune exclusion. As referred, Fc receptors intermediate the connection between cellular and humoral immune systems, by allowing the immunoglobulins to bond to the effector cell surface and exercise their signaling and regulating functions in the intracellular compartment. The different receptors for immunoglobulin support different functions such as maintenance of humoral tolerance and the regulation of innate and adaptive immune response. There are different classes of IgG receptors (FcyR) in lymphocytes currently described with different purposes: phagocytosis (FcyRI or CD64), phagocytosis and regulation (inhibiting) of the antibody production (FcyRII or or CD32), phagocytosis and *antibody-dependent cell-mediated cytotoxicity by NK cells (*FcyRIII or CD16). FcyRII and III have two isoforms, A and B (Rosales & Uribe-Querol, 2013; Tang, Sampson, Dreitz, & McCall, 2001; Tizard, 2013).

Fc receptors have different binding affinities to IgG and signaling pathways. FcyRI has an higher affinity for IgG when compared to the others receptors, being capable of binding to monomeric IgA while the others are only capable of binding to aggregated IgG or immunocomplexes(Allen & Seed, 1989). Whereas IgG_1 and IgG_3 binds to all 3 FcyR, IgG_4 binds only to FcyRII and FcyRIII and IgG_2 uniquely FcyRII. Furthermore IgG_1 and IgG_3 are likely to be induced in response to protein antigens while IgG_2 and IgG_4 respond to polysaccharide antigens. Finally when IgG binds, and concerning the signaling pathways, FcyRI, IIA and IIIA transduce an activating signal, FcyRIIB transmits an inhibitory signal and FcyRIIIB to the absence of one, balancing the immune-response activity (Falk Nimmerjahn & Ravetch, 2007; Schroeder & Cavacini, 2010). All other FcRs selectively interact with the antibodies in the form of immune-complexes (Nimmerjahn & Ravetch, 2007).

IgG plays an important part on antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Bergeron et al., 2014). The ADCC works by binding of the antigenic-antibody immune-complex to activating and inhibitory FcyRs, targets the cell destruction by activate effector cells such as NK cells or monocytes, while the complement-dependent cytotoxicity clears the targeted pathogens by activating the first protein

of the complement cascade (C1q) that binds to the antibody-antigen complex, and that comprises opsonization, chemotaxis and cell destruction (Charles A Janeway, Travers, Walport, & Shlomchik, 2001b). They also have an active role in neutralization of toxins and viruses (Schroeder & Cavacini, 2010).

Contrarily to IgA, the IgG measurements are not significantly correlated with the age of the animal (HogenEsch et al., 2004).

IgG is produced in a delayed response to antigens that leak through the mucosal barrier, consequent of its increased permeability. Its specificity and durability in the serum allows IgG to be an indicator of a previous infection or vaccination, being one of the most used antibodies in clinical diagnostic and researches.

ii.a. IgG in CE dogs

Maeda et al. (2013) reported that, concerning the levels of IgG levels in dogs with chronic enteropathy and healthy dogs, there is no significance difference between the two groups. Also, another study verified that the lesions in Histiocytic ulcerative colitis of the Boxer, show accumulations of IgG+ plasma cells, as well as MHC II + cells, macrophages and granulocytes (Tizard, 2013).

2.3 Modulation of the antibody activity

Different pathways are followed according to the proportion of activating and inhibitory FcRs and their affinity to the ligands. The activated complement components such as C5a is reported to upregulate the activating FcRs, as well as the bacterial endotoxin LPS (one of the main components present in the outer membrane of almost all gram-negative bacteria) and Th1 cytokines, contrarily to Th2 cytokines that cause the downregulation of the activating FcRs and an increment in the FcyRIIB (Nimmerjahn, 2005; Nimmerjahn & Ravetch, 2006; Pricop et al., 2001).

2.4 Immunoglobulins as biomarkers in this study

All things considered, after induction in the MALT, the plasma cells synthetize specific antibodies into the mucosal surfaces and into the circulation in response to the primary antigens. As these productions are consequence of an antigenic insult and a disturbance of the homeostasis resulting in inflammation, it is important to evaluate if the measurement of immunoglobulins in the serum is significantly correlated with the development of inflammatory processes, and as so if it is accurate to use them as biomarkers of inflammatory diseases such as chronic enteropathy.

SECTION 3 – EXPERIMENTAL STUDY – ANTI-BSA ANTIBODIES MEASUREMENT IN DOG'S SERUM

1. BACKGROUND

In humans the link between inflammatory bowel disease (IBD) and dietary habits is well established and confirmed by several epidemiological and experimental studies. Marion-Letellier and others assessed human dietary components and IBD, reaffirming the connection between nutrients as an etiological factor on altering the innate immune response and its significance on the pathogenesis of the disease (Marion-Letellier, Amamou, Savoye, & Ghosh, 2019; Marion-Letellier et al., 2016). In animals, experimental studies are lacking and substantiated data that could elucidate the scientific community about the impact of the dietary components on triggering the immune response are needed.

Dietary antigens commonly contact in high concentrations with the intestinal mucosa, being the mucus and mucosal surface the primary barriers against bacterial and food antigens. In dogs with chronic enteropathy (CE), a disrupted intestinal barrier may result in increased passage of macromolecules, which might over-stimulate the systemic immune system. The positive clinical response to dietary changes observed in food-responsive enteropathy (FRE) supports the diet as a possible trigger of immune system over-activation and as a factor of IBD etiopathogenesis.

Bovine albumin is a common component of animal diet, and the detection of circulating anti-Bovine Albumin IgA and IgG (anti-BSA IgA and IgG respectively) might be a good indicator of intestinal barrier disruption and over-stimulation of the systemic immune system. Furthermore, anti-BSA IgA and IgG might be active actors of immune-mediated food intolerance. Studies about the detection of anti-bovine serum albumin antibodies (anti-BSAA) in dogs with CE are currently lacking.

This study aimed at:

- measuring and comparing the serum anti-BSA antibodies values (IgA/IgG) in healthy or CE-affected dogs.
- assessing possible relations between the anti-BSA levels and the clinical scores.

2. MATERIAL & METHODS

Animals

The present study was conducted using a previously developed database concerning CE-affected and healthy dogs, completed between october 2016 and july 2018 at the Frégis « Centre Hospitalier Vétérinaire », (Paris, France) and the Oniris « Centre Hospitalier Universitaire Vétérinaire » (Nantes, France).

2.1

CE-affected dogs were client-owned dogs with chronic (>3 weeks) gastro-intestinal signs. Throughout their lifetime they had been fed with standard diets containing bovine proteins but after clinical consultation and/or before inclusion in the study they were fed with hydrolyzed bovine-free diets for at least two weeks. Inclusion criteria for enrollment were dogs with CE that did not respond to diet and antibiotic trials, and required an immunosuppressive treatment. The available information about these dogs included breed, age and gender, report of the main complains, clinical signs and CEECAI score at the time of diagnosis, and response to treatment.

Concerning healthy dogs (control group), inclusion criteria was set as: absence of gastrointestinal (GI) signs and currently fed with a standard dry diet. Control dogs included are mainly experimental dogs housed in Oniris, in an animal facility under the control of the Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation. One client-owned dog was also included in the control group.

For client-owned dogs, the owners were informed and filled and signed a letter of consent accepting the collection of a blood sample. All procedures performed in this clinical assay take part of an Internal Medicine research project leaded by Dr. Hernandez. The protocol was validated by the "Comité d'Éthique en Recherche Clinique et Epidémiologie Vétérinaire d'Oniris" (CERVO), ensuing the project number CERVO-2016-13-V validated on September 2016 – Appendix A and B.

2.2 IgA and IgG Dosing Techniques – indirect ELISA

Anti-BSA Antibodies (IgA/IgG) measurement was performed in serum samples using species-specific home-made ELISAs.

In detail, blood samples were collected from the jugular vein in dry tubes and allowed to clot. Serum was obtained after centrifugation at 1000 g at 4°C for 15 minutes, aliquoted in small tubes of 500 µl and frozen at -80°C until the day of the assay.

For the assay, BSA antigen is stably coated on the solid support MAXISORP® 96 well-plate (NUNC 222404). This is possible thanks to the plastic capacity to bind proteins. The working

coating solution composed of BSA in a carbonate buffer (pH 9,6) is prepared by dilution from the 500µg/ml stock solution to a 5 µg/ml final concentration. Uncoated wells (buffer alone) are also kept to check the non-specific adsorption of the serums on the plate, reflecting non-specific signals.

After washing and saturation steps, serums are added (50µl/well) on both coated and uncoated wells and incubated for 2h, at room temperature, leaving some 'blank' wells without serum. In order to adapt and to make this laboratory test accurate, five serial dilutions of each serum were prepared; the reference serum dilution started from one in twelve and a half and the experimental serum ones from one in eight, both ending at one in fifty thousand. These diluted serums were deposed on both coated and uncoated wells, with 50µl/well in duplicate.

After washings, the secondary/detection antibody is added to the wells. Two HRP (Horseradish Peroxidase)–conjugated detection antibodies were used: anti-dog IgA or anti dog-IgG antibodies. The colorimetric substrate (TMB) is mixed with hydrogen peroxide (H_2O_2) in same proportions and 100µl are added per well. In the presence of the enzyme peroxidase, an enzymatic reaction occurs turning the color of the wells blue.

After adding the stop solution (H_2SO_4) after 28 minutes and 5 minutes, respectively, the wells turn to yellow and the plate is read in the Fluostar spectrophotometer which measures the absorbance at 450nm and 540nm. The optical density (OD) is directly related to the intensity of the reaction between the antigen and the antibody, and as that, to the amount of immunoglobulins.

With the intent of easing and simplifying the comparison and working of the data, dilution's log titers were converted in arbitrary units (AU) which is a relative unit of measurement that reflects the ratio of the amount of substance, intensity, or other quantities to a predetermined reference measurement - in this research, the titer of specific IgA and IgG in the serums – Appendix C and D.

2.3 Statistical Analyses

All of the collected data was registered with Microsoft Excel® and analyzed with PRISM® Software. Numerical data is expressed as average, median and range. For analyzing differences between independent variables - (AU) in IBD and healthy dogs - a Mann-Whitney non-parametric test was performed. We also used the Spearmen test to investigate correlation between two parameters, the CCECAI clinical score and the Ig values.

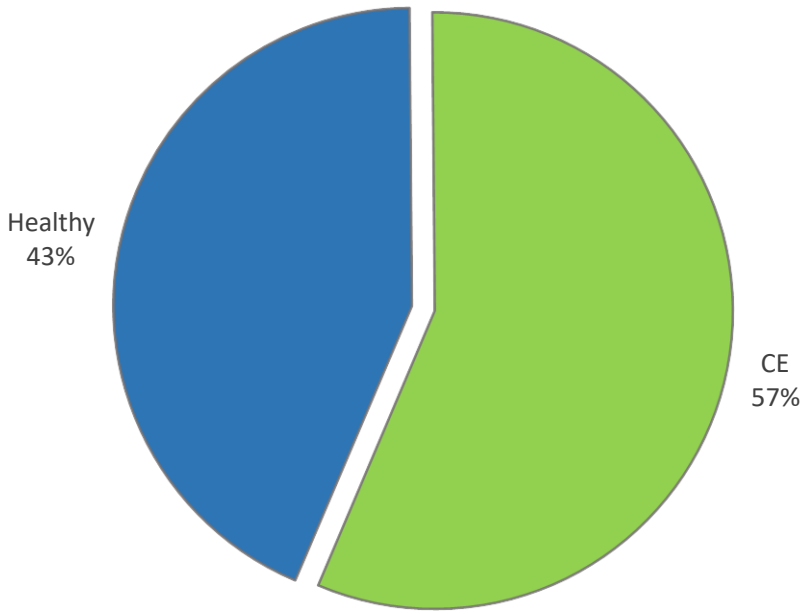
All of the statistical tests comprehend a 95% confidence interval. A significance level of $p<0,05$ was considered, meaning that when p-value $\leq 0,05$ the results are considered statistically significant.

3. RESULTS

3.1 Characterization of the population included

A total of 23 animals were included (Figure 5). The control group was composed of 10 dogs (composing 43% of the analyzed group) while the CE-group had 13 dogs (accounting for 57% of the dogs).

Figure 5 - *Distribution of the population by health status*



3.1.1 Control Dogs

Totaling 10 animals, the control group included 9 animals from the Oniris experimental kennel and 1 client-owned dog from the CHVFrégis.

The median age was 5-year-old, ranging from 3 to 7 year-old. The group of dogs sampled from Oniris experimental alimentation kennel embodies two distinct breeds, Beagle and Briard, with 5 and 3 years respectively. The dog from CHV Frégis was a cross breed dog and was sampled in the course of a routine Internal Medicine consultation.

Concerning breed distribution, the most prevalent breed was the Beagle (5), followed by the Briard (4) and a Cross breed (1) - Table 2.

Table 2 - *Characterization of Control population (n=10)*

Dogs	Breed	Age	Gender
H1	Cross breed	7	MC
H2	Beagle	5	FS
H3	Beagle	*5*	FS
H4	Beagle	5	FS
H5	Beagle	5	FS
H6	Beagle	5	FS
H7	Briard	3	FS
H8	Briard	3	FS
H9	Briard	3	FS
H10	Briard	*3*	*FS*

H, healthy; MC, male castrated; FS, female spayed

3.1.2 CE/IBD Dogs

The CE group was composed by 13 client-owned dogs with clinical signs of CE - Table 3.

These dogs were presented in consultation with chronic digestive clinical signs of different grades, leading to the development of several exclusion tests in order to confirm a potential suspicion of chronic enteropathy. All the dogs were previously submitted to a hydrolyzed diet trial and to an antibiotic trial. Upper and lower endoscopic examinations of the gastrointestinal tract were performed under general anesthesia. Biopsies from stomach, duodenum, ileum, and colon were taken to exclude neoplastic disease and confirm inflammatory infiltrate. Having the histological confirmation of gastro-intestinal inflammation, these animals were categorized as IBD dogs instead of CE Dogs. In the process, blood samples were obtained.

The median age was 7-year-old and the average was 7.5, ranging from 1 to 16-year-old. Breeds included in the IBD population were Cavalier King Charles (1), French Bulldog (2), Briard (1), Teckel (1), Continental Toy Spaniel (1), German Shepherd (1), Rotweiller (1), Yorkshire Terrier (1), JackRussel Terrier (1), West Highland Terrier (1) Golden Retriever (1) and a cross-breed (1). All of them went through blood sampling. Simultaneously, they were scored according to the Canine Chronic Enteropathy Activity Index scoring system – CCECAI.

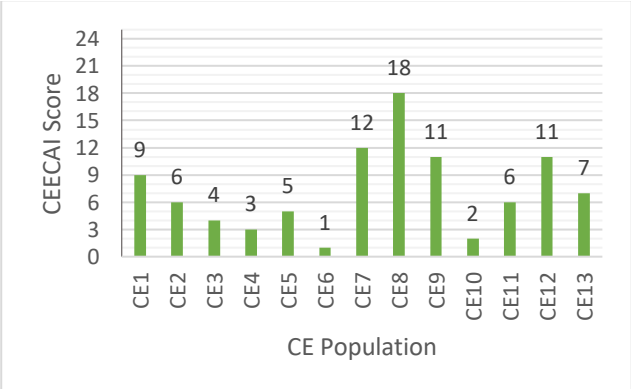
Table 3 - *Characterization of CE/IBD population (n=13)*

Dogs	Breed	Age	Gender
CE1	Cavalier King Charles	3	M
CE2	Cross breed	16	FC
CE3	French Bulldog	4	M
CE4	Briard	6	FC
CE5	Teckel	1	F
CE6	Continental Toy Spaniel	8	FC
CE7	German Shepherd	7	M
CE8	Rottweiler	3	M
CE9	Yorkshire Terrier	13	F
CE10	Jack Russel Terrier	14	MC
CE11	French Bulldog	4	MC
CE12	West Highland White Terrier	11	M
CE13	Golden Retriever	7	MC

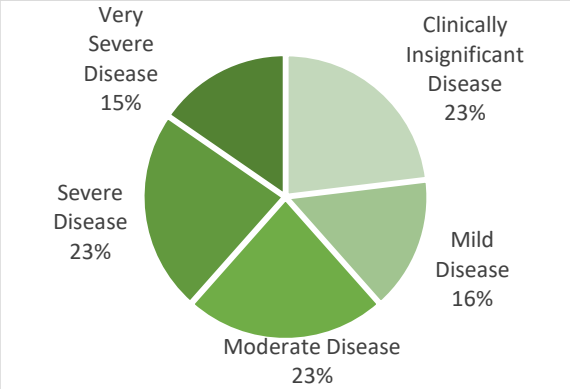
CE, Chronic enteropathy; M, male; MC, male castrated; F, female; FS, female spayed;

Each individual was scored using the CCECAI Index and classified accordingly, upon the severity of the disease, in clinically insignificant (n=3), mild (n=2), moderate (n=3), severe (n=3) and very severe disease (n=2) - Graph 1 and 2, respectively. In the group, scores observed went from 1 to 18, with an average score of 7.3 and a median of 6.

Graph 1 - *Distribution of the population according the CCECAI*



Graph 2 - *Distribution of the population according to the severity of disease*



3.2 Serum anti-BSA IgA and IgG

The results of quantification of anti-BSA IgA and IgG are presented in detail in Appendix E.

It is important to mention that anti-BSA antibodies quantification was not found to be accurate for H3 and H10 because the curves we obtained did not fit typical curves as shown in appendix D. They were thus excluded from the following comparisons.

3.2.1 Comparison between the serum values of IgA in Healthy and IBD Dogs

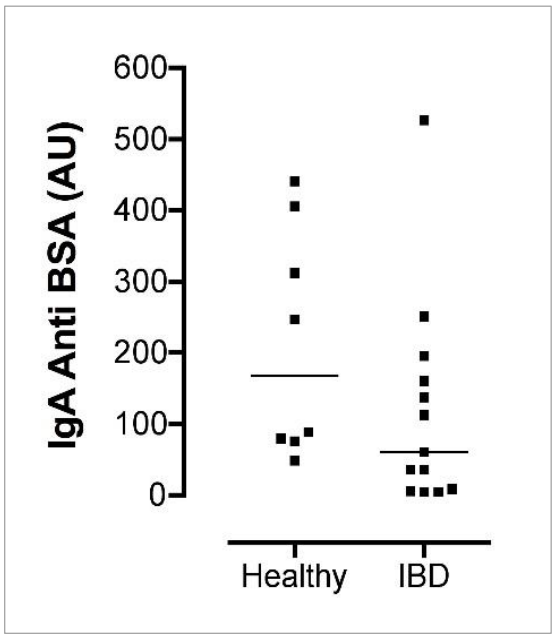
With the aim of better describing and comparing the serological values of circulating IgA in healthy and IBD populations, the average, median, minimum and maximum values in both groups are registered in Table 4.

Table 4 *- IgA serial concentrations (AU) measured in Healthy and IBD dogs*

	Average	Median	Minimum	Maximum
Healthy (n=8)	212,5	168	49	441
IBD (n=13)	118,8	61	5	527

The Mann-Whitney non-parametric test performed showed no significant difference (p= 0,1195) in both healthy and IBD groups (Graph 3).

Graph 3 *- IgA concentrations in Healthy and IBD dogs.*



3.2.2 Comparison between the serical values of IgG in Healthy and IBD Dogs

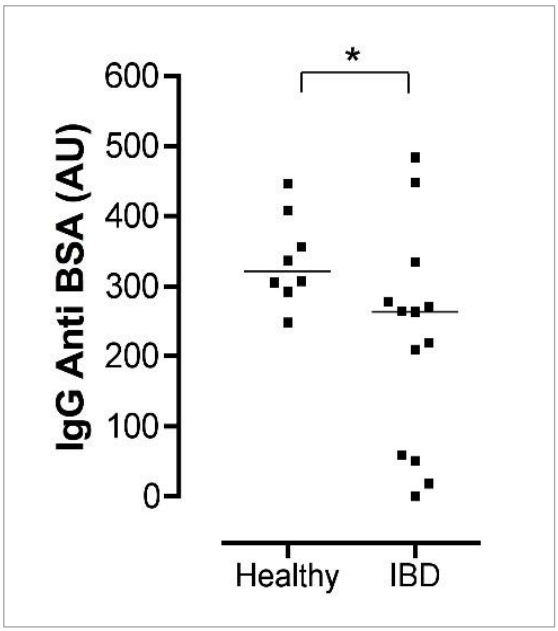
The same procedure was made for IgG values, in both healthy and IBD groups. Results are registered in Table 5.

Table 5 - *IgG serial concentrations (AU) measured in Healthy and IBD dogs*

	Average	Median	Minimum	Maximum
Healthy (n=8)	337,4	322	248	446
IBD (n=13)	222,9	263	0	484

The Mann-Whitney non-parametric test revealed a statistically significant difference between healthy and CE groups (Graph 4). In fact, the measured serological IgG concentrations were higher in healthy dogs when compared to CE dogs (p=0,0465). The IBD group, when compared to the healthy one, showed a lower median value and a higher dispersion between individuals.

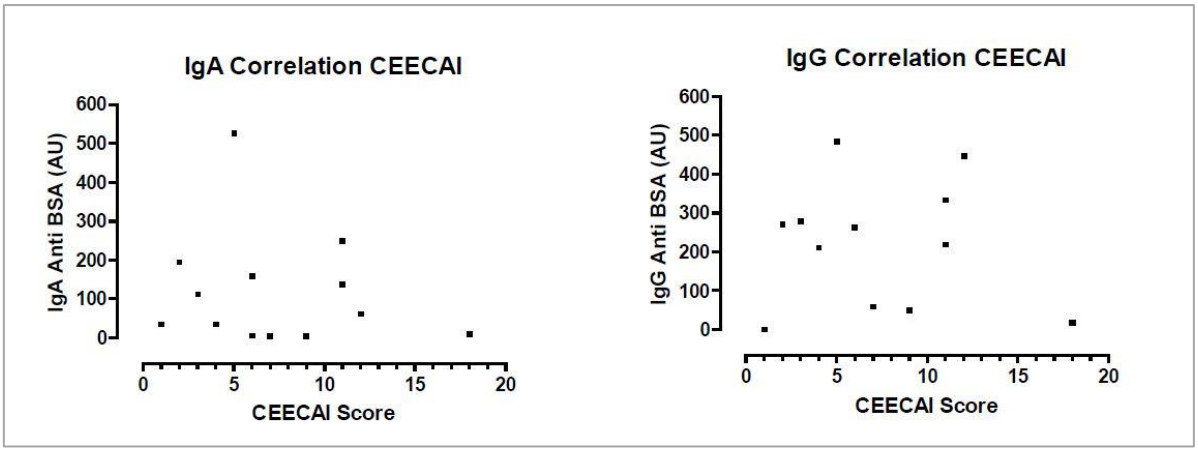
Graph 4 *- IgG concentrations in Healthy and IBD dogs. Note that (*) incomes that p<0,05, reflecting the statistically different medians observed in healthy and IBD groups.*



3.3.3 Correlation of CCECAI clinical score to the IgA values obtained and IgG values obtained

In order to check whether the severity of the disease was correlated to the anti-BSA IgG levels, dogs were classified according to their CCECAI clinical score (Graph 5). Following the Spearmen test, we found no significant correlation between the CCECAI clinical score and the measurement of both serical immunoglobulins measured. For the IgA and IgG, p values observed were 0,5634 and 0,9715 respectively.

Graph 5 - *Correlation between CCECAI Score and the concentrations of IgA and IgG*



DISCUSSION

Up to the present day, there are very few studies relating the values of anti-bovine serum albumin antibodies and the capacity to develop a competent and efficient humoral immune response in dogs with chronic enteropathy, as well as their potential of being adequate biomarkers for leakage in chronical gastrointestinal diseases.

The current project aimed to measure anti-BSA IgA and IgG concentrations in order to assess their potential value as early biomarkers of leakage in dogs with chronic enteropathy. This study did not show significant difference in Anti-BSA IgA levels but did show significant difference in Anti-BSA IgG between healthy and IBD dogs. Anti-BSA IgG level was higher in healthy dogs when compared to dogs with IBD. Therefore, Anti-BSAA do not seem to be good early biomarkers of disease.

Based on the achieved results, IgA and IgG serological concentrations did not show correlation with the severity of clinical signs evaluated with the CCECAI index. Thus, anti-BSA IgA and IgG did not show clinical relevance.

No significant differences were found for anti-BSA IgA levels between healthy and the affected individuals. The measurements observed in each group were equally dispersed between higher and lower values of Anti-BSA IgA. A possible explanation may be that IgA levels are not affected by bovine antigens, meaning that they may not be a trigger for the production of Immunoglobulins A.

Concerning the IgG serological titers, results suggest that IBD dogs have lower IgG circulating levels when compared to healthy dogs. A possible explanation relies on the diet protein source, which can contain bovine extracts. Assuming that diseased dogs had previously contacted with bovine-proteins, another possibility is that dogs with chronic enteropathy are not able to produce enough immune-globulins, showing a poor humoral immunity towards bovine antigens. Maeda et al, 2013, had already identified that total fecal and duodenal IgA were decreased in dogs with IBD, however, IgA and IgG serical levels were similar in diseased and healthy dogs. Our study did not assess fecal and duodenal specific Anti-BSA immunoglobulins but serical IgG was assessed to be lower in IBD dogs. Both studies had shown in different ways that dogs with IBD seem to have lower immunoglobulin levels, reinforcing their poor ability to develop an efficient humoral immune response.

Studies about the use of anti-BSAA as potential biomarkers of a disrupted immune-response in dogs with IBD are lacking, but since a significant difference was observed suggesting that IBD dogs have lower IgG circulating serological values comparing to those measured in healthy dogs, further studies would be important to assess if low-IgG levels can be related with a poor or abnormal general humoral immune response in dogs with IBD.

Apart from IgA and IgG serum values, it would be important to asses IgM values. During the course of a humoral response, and throughout the differentiation of a B cell, the class of the immunoglobulin produced may change from IgM to a major class like IgG, IgA and IgE in response to an activation signal and specific cytokines (Tizard, 2013). Another hypothesis is that this “Class Switch Recombination” process may influence the values measured of IgG since the low levels observed in CE dogs may not be consequent of their poor ability to produce IgG in response to bovine protein but due to an abnormal class switching recombination processing.

Other than IgM, it could have been interesting to measure the IgE levels. Since both groups have historically contacted or were at the time of the study in contact with bovine-proteins, and recognizing that the IBD group was fed with hydrolyzed bovine-free diets for at least two weeks, it might be important to measure the basal values of IgE in both groups, since allergen-specific IgE is highly produced in response to a dietary allergen such as bovine protein, eliciting an immune response based on hypersensitivity mechanisms (Sheldon et al., 2014).

One of the main limitations for assessing the results could be the small sample size. This can be a major factor affecting the accuracy of the results. It is also important to mention that referring to the IBD group, there is a considerable variation in their age compared to the healthy group. This study could be improved by increasing the sample size and thus minimizing the variations within the population.

In addition, and concerning the IBD group that historically contacted with bovine protein-containing standard diets but were latter fed with an hydrolyzed bovine-free diet for at least two weeks, it would be important to extend the period to which these animals were submitted to the dietary therapy - of bovine-free hydrolyzed protein diet - and to identify in detail the full composition data of all the diets they were submitted throughout their life period, both components and quantities, in order to achieve more accurate scientific data.

This information leads to another important question – are IgG serum values an adequate biomarker for the categorization of the healthy status in inflammatory bowel disease? This study hypothesizes that it could be interesting to perform a future study that submits both healthy and IBD dogs to bovine protein alimentary strict diet and then re-do the measurement of IgG values. Therefore, we could more precisely estimate the potential interest of anti-BSA antibodies as markers of leakage.

CONCLUSION

The laborious and rigorous research for the diagnosis in canine gastroenterology arises the necessity of assessing early biomarkers of leakage that can allow the clinician to make an earlier diagnosis of CE.

Anti-BSAA do not seem to be good biomarkers of “leaky gut”. A possible explanation for the fact that IgG levels are higher in healthy dogs relies on the diet protein source, which can contain bovine extracts. Assuming that diseased dogs had previously contacted with bovine-proteins, other possibility is that dogs with chronic enteropathy are not able to produce enough immune-globulins, showing a poor humoral immunity towards bovine antigens. Similarly to previous studies, these results also disbelieve the use of serologic serum profiles to assess potential dietary antigenicity.

Albeit bovine protein is historically believed to be one of the highest immune triggers in dogs with IBD, these preliminary results supports that anti-BSAA do not seem to be good biomarkers of a “leaky gut”. Further studies are needed to evaluate if low-IgG levels can be related with a poor humoral immune response in dogs with CE.

Overall, this work contributed to increase the current knowledge of the leaky gut, highlighting the need of further immunological studies to better understand the immune pathways beneath chronic GI disease in dogs.

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APPENDIX

APPENDIX A – Patient Consent Form

Patient Consent Form

Your dog suffers from vomiting, diarrhea, borborygmus, abdominal pain, nausea and/or weight loss. We suspect an Inflammatory Bowel Disease (IBD). To confirm this diagnosis, endoscopic examination and digestive biopsies are indicated. Endoscopic examination is useful to visualize the different parts of the digestive tract and allow digestive biopsies. The examination is performed under general anesthesia. The total duration of the procedure is of approximately 60-90 minutes for an upper and lower gastro-intestinal tract endoscopy. Like any medical act, even conducted with competences and in the respect of the Science, digestive endoscopy carries a risk. The current monitoring of the anesthetic and waking periods allow quick detection of any abnormalities. Also, serious complications of anesthesia have become rare. Similarly, endoscopic examination is performed with appropriate equipment (pediatric endoscope) by an experienced veterinarian. Serious complications such as perforations, heavy bleeding or infections have become exceptional.

The treatment of IBD is most often based on the administration of corticosteroids with potentially significant long-term side effects. By being included in the study, we suggest to try a more natural treatment approach with less known side effects.

Concretely, more and more scientific information tends to involve bacteria leaving in the intestine (scientifically called the microbiota) in the appearance and maintenance of intestinal inflammation. Studies show that intestinal microbiota is disrupted in IBD patients and restoration of a normal microbiota may cause the signs to regress in a number of cases. The restoration of a microbiota involves a normal stool transplant. This practice is very well documented in human medicine and is the treatment of choice for certain intestinal diseases.

We therefore propose to participate in a study whose objective is to know the clinical effectiveness of this treatment in dogs with IBD. By agreeing to participate, your dog will be randomly assigned to one of 2 groups: 1) Treated group: he will receive a stool preparation from 2 specially selected donors or 2) Control group: he will receive a preparation of his own prepared stool. There is no way to know which group your dog is assigned to before the end of the study.

By participating in this study, we ask you to schedule follow-up with the internal medicine service at 10, 30 and 90 days after treatment. In return for your involvement, the funding of the study covers the costs of anesthesia, endoscopy, histopathology, fecal transplantation and clinical monitoring at 10 days, 1 month and 3 months.

"I, the undersigned ..... (Surname, name), residing ..... (Address) at ..... (Locality) acknowledges having been informed by .... (Surname, name) of all the procedures involved in the Microbiota Fecal Transplantation clinical study in which I freely agree to include the following animals of which I am the owner * or the holder * (specify below available identifiers: Optional name, identification number, species, breed and age:

I certify that I have understood the expected benefits and the potential risks listed on the back of this page, related to the implementation of this study and which were clearly stated to me by .... (Last name First Name).

I have noted that participation in the clinical study does not involve any additional costs borne by me nor is it accompanied by associated remuneration.

I have been told that I have the right at any time and for whatever reason to remove my animals from the study.

I authorize Oniris research teams and / or their partners to use the data and samples of my animal anonymously to improve the state of scientific and technical knowledge on the subject. However, I consent to the lifting of anonymity if it is requested by the public authorities.

I declare having had from .... (Name First Name) all the answers to my questions.

Done at ....., The .... 20 ..

Signature of the owner * of the animals

or his representative *

Expected benefits	Possible constraints
• Regression of digestive signs • Avoid the use of corticosteroids • No known side effects of treatment • The funding of the study covers the costs of anesthesia, endoscopy, histopathology, fecal transplantation and clinical monitoring at day 10, 30 and 90.	• Need to stagger endoscopic investigation for one week to allow transplant preparation • Clinical follow-up at day 10, 30 and 90 at the internal medicine servcice of Oniris • Stool collection at day-7, D30 and D90 (collected during spontaneous defecation) • Collection of blood on day 0, 10, 30 and 90. • Performing 7 additional digestive biopsies during endoscopy for further analyses. • In the event of no improvement in the patient's state of health, delay in setting up a corticosteroid treatment from 10 days to 1 month

APPENDIX B – Ethical Approval

Oniris' ethics committee for
clinical and epidemiological veterinary research
Atlanpole- La Chantrerie
44307 Nantes Cedex 03
FRANCE
e-mail: secrétariat.cervo@oniris-nantes.fr
Tel: +33-240687718

Nantes, January 30th 2017

To Mr Juan HERNANDEZ and Mrs Blandine LIEUBEAU,
Unité de Recherche IECM - Oniris

Dear investigators,

Thank you for having submitted your project entitled "Rôle des récepteurs TLRs dans les entéropathies inflammatoires chroniques idiopathiques du chien" to the Oniris' Ethics Committee for clinical and epidemiological Veterinary Research (CERVO).

Your submission was reviewed during the Committee meeting held on 09-14-2016.

Considering submitted documents, the way animal welfare was taken into account and answers given to questions from our experts, I am pleased to inform you that CERVO members approved your protocol. Your approval number is CERVO-2016-13-V.

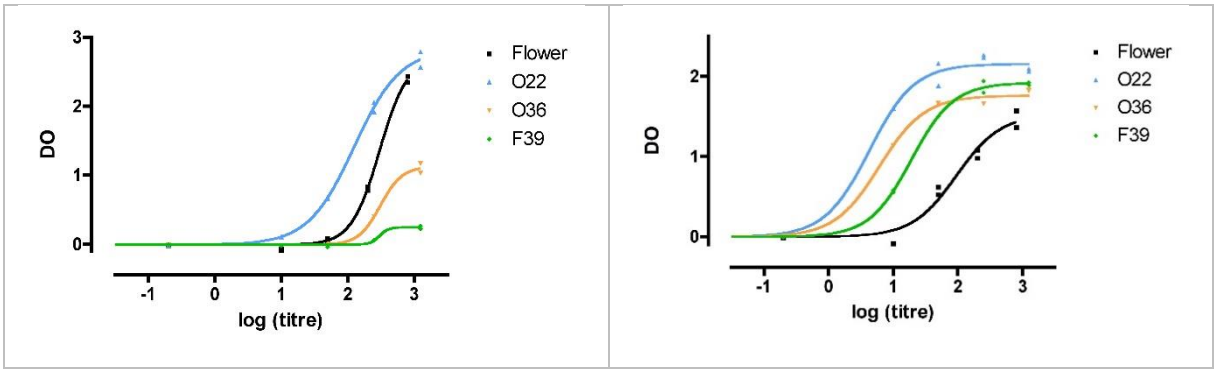
If your need to modify the protocol in any way, (i.e. change in clinical procedure, change in the number of subjects, extension of time…) an amendment will have to be submitted to the CERVO before implementation of any change.

Solenn Kerloc'h
Chair of the CERVO

APPENDIX C - Reference (Flower) and Experimental Serum Dilutions

Flower Serum		
Dilution of the serum	Titer DO (AU)	log (Titer)
1/12,5	800	2,90308999
1/50	200	2,30103
1/200	50	1,69897
1/1000	10	1
1/5000	2	0,30103
1/50000	0,2	-0,69897
Experimental Serums		
Dilution of the serum	Titer DO (AU)	log (Titer)
1/8	1250	3,09691001
1/40	250	2,39794001
1/200	50	1,69897
1/1000	10	1
1/5000	2	0,30103
1/50000	0,2	-0,69897

APPENDIX D - IgA and IgG serum titers according to their optic densitometry in three of the twenty-four analyzed animals



APPENDIX E - Measured values of IgA and IgG in Healthy (n=10) and CE dogs (n=13)

Healthy	IgA Anti-BSA (AU)	IgG Anti-BSA (AU)
H1	80	307
H2	89	248
H3	293	404
H4	76	408
H5	247	356
H6	406	305
H7	312	337
H8	49	292
H9	441	446
H10	181	278
CE	**IgA Anti-BSA (AU)**	**IgG Anti-BSA (AU)**
CE1	5	51
CE2	161	263
CE3	36	210
CE4	113	278
CE5	527	484
CE6	36	0
CE7	61	448
CE8	9	18
CE9	251	334
CE10	196	270
CE11	6	264
CE12	138	219
CE13	5	59