UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

OLD ENEMIES, NEW DIFFERENTIALS: CANINE LEISHMANIOSIS AS A CAUSE OF REFRACTORY ENTERITIS

INÊS DIAS LOPES DE OLIVEIRA BRANCO

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2024

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INÊS DIAS LOPES DE OLIVEIRA BRANCO

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VELHOS INIMIGOS, NOVOS DIFERENCIAIS: LEISHMANIOSE CANINA COMO CAUSA DE ENTERITE REFRATÁRIA

Resumo

A enteropatia crónica refratária (ECR) é responsável por uma percentagem pequena, mas não insignificante, de casos em gastroenterologia canina. A diarreia crónica, embora incomum, é um sinal clínico associado à leishmaniose canina e, ao longo do tempo, os relatos de casos com sinais exclusivamente gastrointestinais, particularmente diarreia, têm sido crescentes.

O objetivo deste trabalho foi tentar estabelecer uma relação entre ECR e leishmaniose canina. Sendo Lisboa uma área endémica para este parasita, hipotisámos que haveria até 20 a 30 por cento de cães com ECR positivos em imunohistoquímica para o protozoário endémico *Leishmania infantum* em biópsias digestivas.

Foi realizado um estudo retrospetivo tendo como base casos de ECR submetidos a gastroduodenoscopia ou colonoscopia com biópsias, entre janeiro de 2018 e outubro de 2022, no Hospital Escolar Veterinário da Universidade de Lisboa. Os casos foram revistos quanto a sinais clínicos, alterações laboratoriais, tratamento, progressão clínica, serologia e reação em cadeia da polimerase (PCR) para *Leishmania*. As amostras de biópsia foram analisadas histologicamente para confirmar a presença de inflamação crónica e foi realizada uma técnica de imunohistoquímica para *L. infantum* para cada uma delas.

Foram incluídos onze casos. O sinal clínico principal foi diarreia ou fezes moles apresentado por todos (11/11; 100%) os cães, seguido de perda de peso e vómito presentes em 9/11 (82%) e 6/11 (55%) casos, respectivamente. A serologia para *Leishmania* estava disponível para 5/11 (45%) casos e apenas 1/11 (20%) apresentou um resultado positivo. Amostras de duodeno do cão positivo foram submetidas à técnica de PCR para *L. infantum* e os resultados obtidos foram negativos. Foram analisadas retrospectivamente amostras de duodeno de 8 dos 11 cães, amostras de íleo de 4 dos 11 cães e amostras de cólon de 6/11 cães. Todas apresentaram inflamação crónica com componente linfoplasmocítica na análise histopatológica, contudo todas as amostras foram negativas para *Leishmania* na imunohistoquímica.

Estes resultados salientam que, embora a leishmaniose deva ser incluída como diagnóstico diferencial de diarreia crónica, a deteção do protozoário não foi identificada em imunohistoquímica em nenhum dos casos, questionando o seu papel como agente causal em casos de ECR.

Palavras-chave: Enteropatia Crónica Refratária, Leishmaniose Canina, Imunohistoquímica.

OLD ENEMIES, NEW DIFFERENTIALS: CANINE LEISHMANIOSIS AS A CAUSE OF REFRACTORY ENTERITIS

Abstract

Refractory chronic enteropathy (RCE) accounts for a small but not insignificant percentage of cases in canine gastroenterology. Chronic diarrhea, albeit uncommon, is a clinical sign associated with canine leishmaniosis and, over time, reports of cases with solely gastrointestinal signs, particularly diarrhea, have been increasing.

The aim of this project was to attempt and establish a connection between RCE and canine leishmaniosis. As Lisbon is an endemic area for this parasite, we hypothesized that there would be up to 20 to 30 percent of dogs with RCE that were positive in immunohistochemistry for the endemic protozoan *Leishmania infantum* in digestive biopsies.

A retrospective study was conducted based on RCE cases undergoing gastroduodenoscopy or colonoscopy with biopsies, between January of 2018 and October of 2022 at the Veterinary Teaching Hospital of the University of Lisbon. Cases were reviewed for clinical signs, laboratory findings, treatment, clinical progression, serology and polymerase chain reaction (PCR) for *Leishmania*. Biopsy samples were histologically analyzed to confirm the presence of chronic inflammation and an immunohistochemistry technique for *L. infantum* was performed in each sample.

Eleven cases were included. The primary clinical sign was diarrhea or loose stools presented by all (11/11, 100%) dogs, followed by weight loss and vomiting presented by 9/11 (82%) and 6/11 (55%) cases, respectively. Serology for *Leishmania* was available for 5/11 (45%) cases and only 1/11 (20%) tested positive. Duodenum samples from the positive dog were submitted to PCR for *L. infantum* and were negative. Duodenum samples were obtained from 8 out of the 11 dogs, ileum samples from 4/11 dogs and colon samples from 6/11 dogs. All of them presented chronic inflammation with a lymphoplasmacytic component on histopathology, however, all samples were negative for *Leishmania* on immunohistochemistry.

These results highlight that, although leishmaniosis should still be included as a differential diagnosis of chronic diarrhea, the detection of the protozoan was not identified in immunohistochemistry in any of the cases, questioning its role as a causal agent in RCE cases.

Key words: Refractory Chronic Enteropathy, Canine Leishmaniosis, Immunohistochemistry.

VELHOS INIMIGOS, NOVOS DIFERENCIAIS: LEISHMANIOSE CANINA COMO CAUSA DE ENTERITE REFRATÁRIA

Resumo Alargado

A enteropatia crónica refratária (ECR) é responsável por uma percentagem pequena, mas não insignificante, de casos em gastroenterologia canina. A diarreia crónica, embora incomum, é um sinal clínico associado à leishmaniose canina e, ao longo do tempo, os relatos de casos com sinais exclusivamente gastrointestinais, particularmente diarreia, têm sido crescentes. A leishmaniose canina é endémica em Portugal e a seroprevalência da infeção quase duplicou nos últimos 10 anos. Apesar destas evidências, a leishmaniose não está incluída na investigação para abordar uma diarreia crónica. Estudos anteriores demonstraram que, por vezes, abordagens mais simples e com melhor relação custo-eficácia poderiam ter identificado a etiologia da diarreia crónica. Tal observação levanta questões sobre a possibilidade um cenário semelhante ocorrer se os médicos veterinários realizarem testes de rastreio para *Leishmania* mais cedo em regiões endémicas.

Existe pouca informação sobre a ligação entre enteropatia crónica e leishmaniose em Portugal. Especificamente, se existem casos de leishmaniose com manifestações exclusivamente gastrointestinais em Portugal, uma vez que, tanto quanto é do conhecimento dos autores, não existem atualmente estudos que relatem tais ocorrências. Este estudo tem como objetivo explorar uma possível conexão entre ECR e leishmaniose.

Hipotisámos que haveria até 20 a 30 por cento de cães com ECR positivos em imunohistoquímica para o protozoário endémico *Leishmania infantum*.

Foi realizado um estudo retrospetivo tendo como base casos de ECR seguidos no Serviço de Medicina Interna do Hospital Escolar Veterinário da Universidade de Lisboa, entre janeiro de 2018 e outubro de 2022. Durante este período, os animais incluídos no estudo teriam de ter sido submetidos a uma gastroduodenoscopia e/ou colonoscopia com biópsias, que ficariam conservadas nos arquivos do laboratório de patologia da universidade, e teriam de ter passado pelos três pilares do tratamento para a enteropatia crónica – alteração para uma dieta com nova proteína ou dieta hidrolisada, tratamento direcionado para a disbiose intestinal idiopática e tratamento com imunossupressores. As amostras de biópsias foram analisadas histologicamente segundo parâmetros morfológicos e inflamatórios com base nas *guidelines* da *World Small Animal Veterinary Association* para confirmar a presença de inflamação crónica e foi realizada uma técnica de imunohistoquímica para *Leishmania infantum* para cada uma delas. Como controlo positivo, foram utilizadas lâminas com amostras de uma lesão da cavidade oral de um gato naturalmente infetado por *Leishmania infantum*. Através da plataforma *Guruvet*, os casos

foram revistos quanto a sinais clínicos, alterações laboratoriais, tratamento, progressão clínica, serologia e reação em cadeia da polimerase (PCR) para *Leishmania infantum*.

Foram incluídos 11 animais no estudo. A mediana da idade ao diagnóstico foi de 5 anos. Dois cães (2/11; 18%) eram sem raça definida, 2/11 (18%) eram Yorkshire Terriers e havia um (1/11; 9%) animal de cada uma das seguintes raças: Cão de Água Português, Lulu-da-Pomerânia, Golden Retriever, Pastor Alemão, Dogue Alemão, Pinscher e Boxer.

O principal sinal clínico de apresentação foi diarreia ou fezes moles apresentado por todos (11/11; 100%) os cães, seguido de perda de peso e vómito presentes em 9/11 (82%) e 6/11 (55%) casos, respetivamente. Os principais sinais clínicos refratários foram diarreia ou fezes moles, vómito e hematoquézia, apresentados por 10/11 (91%), 6/11 (55%) e 6/11 (55%) animais, respetivamente.

A serologia para *Leishmania* estava disponível para 5/11 (45%) casos e apenas 1/11 (20%) apresentou um resultado positivo. Amostras de duodeno do cão positivo foram submetidas à técnica de PCR para *Leishmania infantum* e os resultados obtidos foram negativos. Foram analisadas retrospetivamente amostras de duodeno de 8 dos 11 cães, amostras de íleo de 4 dos 11 cães e amostras de cólon de 6 dos 11 cães. Todas apresentaram inflamação crónica com componente linfoplasmocítica na análise histopatológica, contudo todas as amostras foram negativas para *Leishmania* na imunohistoquímica.

Tal como estabelecido pelos parâmetros de inclusão, todos os animais (11/11; 100%) foram submetidos a alteração da dieta, tratamento direcionado para a disbiose e tratamento com imunossupressores. Sete cães (7/11; 64%) fizeram hemograma até um mês antes da endoscopia e 8/11 (73%) realizaram análises bioquímicas no mesmo período.

Três (3/11; 27%) dos onze animais morreram, 2/3 (67%) deles foram eutanasiados e 1/3 (33%) morreu por causas desconhecidas. Dos restantes 8/11 (73%), 5/8 (63%) perderam-se durante o seguimento e os outros 3/8 (38%) ainda são seguidos no Hospital Escolar Veterinário.

A análise dos critérios inflamatórios das lâminas demonstrou que os linfócitos e plasmócitos foram as células inflamatórias predominantes na lâmina própria, corroborando com a literatura que aponta a enterite linfoplasmocítica como a forma mais prevalente de enterite. Numa avaliação preliminar dos resultados, os plasmócitos pareceram ter uma maior expressão em termos de severidade em comparação com os linfócitos. Contudo, uma vez que não foi realizada contagem celular durante a análise histológica, não foi possível realizar testes estatísticos para determinar se esta diferença seria significativa. De acordo com a literatura disponível em medicina veterinária, não parece existir um padrão inflamatório predominante, sendo comum observar-se um padrão misto de citoquinas.

A imunohistoquímica foi a técnica utilizada neste estudo, uma vez que já demonstrou ser eficaz para o diagnóstico por vários autores. No entanto, a imunohistoquímica envolve diversas etapas repetitivas e, no caso do laboratório da Faculdade de Medicina Veterinária, dependentes de um operador humano, aumentando a probabilidade de erros técnicos e, consequentemente, de coloração inadequada. No contexto específico deste estudo, observou-se coloração adequada no controlo positivo, enquanto todas as amostras apresentaram ausência de coloração. Portanto, caso esta ausência de coloração não fosse indicativa de resultados negativos mas sim de limitações da técnica, seria de esperar que essas limitações estivessem associadas a etapas pré-analíticas, nomeadamente fixação, processamento e recuperação antigénica.

No contexto específico da leishmaniose canina, a técnica de imunohistoquímica é frequentemente utilizada por diversos autores para o diagnóstico da doença. No entanto, a sensibilidade para detetar amastigotas de *Leishmania* parece variar de acordo com os sinais clínicos apresentados. É sugerido que esta variação de sensibilidade possa ser atribuída a uma carga parasitária muito baixa na pele dos animais assintomáticos. Além disso, foi demonstrado que o PCR possui maior sensibilidade do que a imunohistoquímica na deteção de *Leishmania*, especialmente em tecidos fixados em formol e embebidos em parafina. Contudo, à semelhança do que se verifica na imunohistoquímica, também o PCR apresenta limitações inerentes à técnica, possuindo cada um dos métodos de diagnóstico vantagens e desvantagens que variam conforme o objetivo pretendido. A menor sensibilidade da imunohistoquímica, quando comparada ao PCR, é parcialmente explicada pela necessidade de que o antigénio esteja em perfeitas condições estruturais para que os anticorpos se possam ligar e produzir um resultado positivo. Por outro lado, no PCR, onde ocorre um processo de amplificação, a presença de uma pequena sequência de material genético é suficiente para obter um resultado positivo.

Deste modo, num cenário em que o insulto inicial que desencadeou o processo inflamatório tenha sido a *Leishmania*, mas onde os antigénios já não se encontrem intactos ou a carga parasitária seja muito reduzida, a imunohistoquímica não seria capaz de detetar amastigotas. No entanto, a presença de um fragmento mínimo de material genético de *Leishmania* seria suficiente para que o PCR detetasse a sua presença, resultando num teste positivo. Tendo isto em consideração, é possível que as amostras dos casos refratários deste estudo exibam tais características e beneficiassem da combinação de técnicas e subsequente comparação de resultados, tal como sugerido por artigos que recomendam a utilização do PCR nos casos em que permanece a suspeita de leishmaniose após um resultado negativo na imunohistoquímica.

Assim, e de modo a ultrapassar algumas limitações do estudo, futuras investigações nesta temática devem ser realizadas a nível nacional para se obter uma amostra maior,

mais representativa e menos heterogénea. Idealmente, estes estudos deverão ser prospetivos para reduzir a subjetividade dos resultados através da avaliação dos índices de atividade clínica. O estudo beneficiaria da associação das técnicas de imunohistoquímica e PCR, uma vez que já demonstraram ser complementares. Apesar da imunohistoquímica nos fornecer uma visão contemporânea da relação entre as lesões e a presença ou ausência do parasita no intestino, que constitui o principal objetivo do estudo, seria interessante também obter-se um contexto histórico dos animais em relação à *Leishmania*. Para isso, seria útil realizar serologia e PCR de um órgão hematopoiético, como a medula óssea, para determinar se os animais poderiam ter estado previamente infetados com *Leishmania* ou se estão atualmente infetados mas sem envolvimento gastrointestinal.

Este estudo proporcionou novos pontos de vista sobre a utilidade da imunohistoquímica para o diagnóstico de leishmaniose canina em amostras de biópsia de cães com ECR, questionando a sua utilidade atual. Apesar de se tratar de um diagnóstico menos provável, os resultados obtidos não devem desvalorizar a necessidade de investigar casos de leishmaniose digestiva, especialmente em regiões endémicas, quando acompanhados de resultados clínicos ou laboratoriais que o justifiquem. Este cuidado é particularmente relevante à luz dos resultados positivos relatados em diversos estudos publicados internacionalmente. No entanto, de acordo com os dados do nosso estudo, a *Leishmania* não parece ser um agente causal de enteropatia crónica em casos refratários.

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1 - Traineeship Report

The final curricular traineeship took place between September 5th, 2022 and March 3rd, 2023 at the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine of the University of Lisbon, making a total of 25 weeks. The daily shifts were 7 hours long and two night shifts of 12 hours were also added to the schedule, making a total of approximately 900 hours. Half of the traineeship (3 months) was spent in the internal medicine service and the other 3 months consisted of rotations through the different specialties of the hospital.

The practical component of this master's project was completed during the curricular traineeship, allowing an oral presentation at the ‘’*Encontro de Formação da Ordem dos Médicos Veterinários*’’ which abstract is available in the annexes section (Annex 1).

1.1 - Internal Medicine

Approximately 490 hours were spent in this service. During that time, the trainee was able to follow Professor Dr. Rodolfo Oliveira Leal, Dr. Joana Dias and Dr. Miguel Carvalho in their consultations, during the morning period. It was possible, with supervision, to carry out the initial part of the consultations by collecting the anamnesis, formulating the problem list and performing the physical examination. The differential diagnoses, treatment and prognosis were later discussed with the supervisor. Additionally, during the consultation period, it was possible for the trainee to actively participate in procedures such as the collection of blood and urine samples (venipuncture and cystocentesis, respectively), blood pressure measurement, blood glucose measurements and application of continuous interstitial glucose monitoring devices (Freestyle Libre ®). Twice a week, it was also possible for the trainee to assist complementary exams such as gastroduodenoscopies, colonoscopies, tracheobronchoscopies with bronchoalveolar lavages, rhinoscopies and cystoscopies. Most of these procedures included collecting biopsies. Although less frequent in this service, emergency consultations were also carried out, like cases of diabetic ketoacidosis or ingestion of foreign bodies.

During the afternoon, the doctors from the internal medicine service discussed the clinical cases of the morning appointments as a team and then proceeded to the discussion and communication of exam results to the owners. Periodically, journal clubs were also held in the afternoon where the doctors and trainees would prepare and present an article of one of the standard internal medicine journals, namely Journal of Veterinary Internal Medicine. During the period spent in this service, it was also possible to assist Professor Rodolfo Leal's research project, which aimed to increase student engagement in clinical rotations.

This service allowed the development of medical skills, mainly in chronic cases such as endocrine, gastrointestinal and respiratory diseases. In addition, it allowed the development of critical thinking and the following of a logical order to get to a diagnosis, evaluating all the details of the case. Finally, it was also an opportunity to develop soft communication skills by interacting with both the owners and students from the hospital rotations.

1.2 - General Medicine

A total of 175 hours were completed in this service. In general medicine, it was possible to learn from a lot of different doctors from the clinical staff and the consults ranged from vaccination, deworming and check-up appointments to first and second opinion consultations in all areas, follow-ups, and emergency and life-threatening situations. Similarly to the internal medicine service, an active participation in consultations was also possible here. The trainee had the opportunity to start the consultations by collecting the animal's clinical history and carrying out a physical examination, which would be either supervised or later confirmed by the doctor on duty. Furthermore, when necessary, the trainee could collect blood samples, learn and practice how to do a cystocentesis, help restraining the patient, prepare and administer fluids and medications as well as place catheters. Whenever necessary, according to the clinical signs of the animal, it was possible to assist in complementary diagnostic exams such as radiographs and ultrasounds. In this service, help was also provided to nurses whenever necessary, mainly for restraining animals to apply dressings, removing sutures and staples and collecting various samples. This service provided the necessary insights for daily clinical practice, which will also be the trainees' first contact with the profession. In addition, emergency situations were a fundamental tool to teach quick thinking, focusing on what was strictly necessary at the moment to stabilize the patient.

1.3 - Hospitalization

Approximately 24 hours were completed in the hospitalization service, which consisted of two night shifts. Each clinical case was discussed in detail during two rounds, at the beginning and at the end of each shift. The patient's history and cause of hospitalization were discussed as well as the clinical progression under hospitalization, the treatment plan and prognosis. Twice a shift, the trainee carried out the physical examinations, cleaned each animal box, provided food, water, any medication or fluids needed and, depending on the animal’s condition, walked the patients. When necessary, the trainee also assisted in the

collection of samples for analysis and in the restraining of the animal. In this service, urgent and life-threatening situations also arose, either with critical patients or, overnight, in urgent consultations that may arise at any time. Whenever possible, the trainee assisted in these moments as well.

1.4 - Ophthalmology

Around 35 hours were spent in this service. In ophthalmology it was possible to perform, in addition to the general physical examination, the ophthalmological examination with evaluation of palpebral, pupillary reflexes and menace response, performance of the Schirmer and fluorescein test, measurement of intraocular pressure and evaluation of the anterior and posterior segments, after applying a mydriatic. When necessary, blood was collected for complementary analyses such as FIV/FeLV status. In this service, there were referral appointments, first opinion and follow-ups, although the most observed cases were follow-ups of corneal ulcers.

1.5 - Surgery

Approximately 35 hours were spent in this service. Activities included receiving patients, weighing and performing a pre-anesthetic physical examination in each animal. After that, all scheduled medication was prepared and premedication administered, followed by catheterization of the patient. The animal was taken out of the box shortly before the procedure and then anesthesia induction was performed. This was followed by animal preparation with intubation, trichotomy of the area to be intervened, cleaning and disinfection. The student was able to help with all of the above-mentioned activities. In the operating room, the trainee assisted the surgeon whenever asked. However, the main intervention occurred during the application of the skin sutures to close the incision, at the end of the procedure. It was possible to observe several types of procedures, from soft tissue surgeries to orthopedic and stomatology surgeries. The student was also able to practice performing orchiectomies under the supervision of a surgeon. Finally, in the postoperative period, it was also necessary to monitor the animal until discharge or until hospitalization, when needed.

1.6 - Oncology

About 35 hours were completed in this service. Reassessment, referral and first opinion appointments were all part of this rotation, which also included chemotherapy

treatments twice a week. The student had the opportunity to take part in the physical examination, discussion of the diagnosis and chemotherapy treatment as well as its adverse effects and discussion of the prognosis. In this rotation, fine needle aspiration punctures were often performed as a means of diagnosing different alterations detected on physical examination. In what regards to chemotherapy, the trainee was involved in the reception of the patient as well as it's weighing, catheterizing and blood collection for complete blood count. The preparation and administration of chemotherapy was carried out by the nursing team, with the trainee assisting in monitoring the animal. Through this service, soft skills for delivering bad news and dealing with more challenging circumstances, such as worse prognosis, were developed.

1.7 – Ultrasound / Diagnostic imaging

Approximately 70 hours were performed in this rotation. The student was able to watch several ultrasound scans while restraining the animal. In selected instances, the trainee was allowed to attempt to conduct the ultrasound examination and recognize the various structures, always under guidance and supervision. Most of the ultrasounds seen were abdominal ultrasounds. Moreover, other procedures that required ultrasound guidance were often carried out such as cystocentesis, abdominocentesis, fine needle aspiration (of the liver, gallbladder, spleen and abdominal masses) and Subcutaneous Ureteral Bypass (SUB) flushing. Additionally, by observing the writing of the reports after the exam, the student had the chance to become familiar with the appropriate terminology and vocabulary required for this sort of reports.

1.8 - Infectious Disease Isolation Unit

Approximately 35 hours were completed in this service. In the isolation unit, the student was able to learn both the protocols for handling animals with infectious diseases (e.g. calicivirus, herpesvirus, feline panleukopenia virus, canine parvovirus, feline leukemia virus), always using personal protective equipment, and the protocols for cleaning and disinfecting the spaces occupied by these animals. Before entering the isolated area, all of the patients' food, water and medications were prepared. Once inside, a physical examination was conducted according to the proper protocol, medications were given as needed, biological material was collected as needed and each animal's box was cleaned. After all these procedures, a careful cleaning and disinfection of the space was always carried out. The trainee also had the opportunity to observe discharge consults in this unit as well.

2 - Literature Review

2.1 - Chronic Enteropathy

Chronic enteropathy is defined as a set of persistent or recurrent gastrointestinal clinical signs lasting more than 3 weeks (Hall and Day 2017). Vomiting and diarrhea (small or large intestinal type) are the most common signs. However, borborygmus, hyporexia, abdominal pain, nausea and weight loss are also very frequently reported (Hall and Day 2017).

According to treatment response, chronic enteropathy has been historically subdivided into food-responsive enteropathy (FRE), antibiotic responsive enteropathy (ARE), immunosuppressant-responsive enteropathy (IRE) and non-responsive enteropathy (NRE) for those dogs who do not respond to any of the above mentioned therapies. Additionally, dogs with loss of protein across the intestine are often categorized as having protein-losing enteropathy (PLE), emphasizing the less favorable prognosis (Dandrieux 2016).

It is important to point out that nowadays, the nomenclature "antibiotic-responsive enteropathy" is transitioning to "idiopathic intestinal dysbiosis" (Jergens and Heilmann 2022) or microbiota-related modulation-responsive enteropathy (Dupouy-Manescau et al. 2024). This change reflects the growing interest and support by clinical research in alternative treatments such as prebiotics, probiotics and synbiotics to control bacterial populations (Rossi et al. 2014; White et al. 2017; Rossi et al. 2018; Cerquetella et al. 2020) as well as fecal microbiota transplantation (Cerquetella et al. 2020). These options should be taken into account since the effect of antibiotics in the intestine can have a long-lasting effect on its microbiome (Suchodolski et al. 2009; Manchester et al. 2019; Werner et al. 2020) and the majority of dogs who initially respond to an antibiotic treatment will most likely relapse when treatment is interrupted (Dandrieux and Mansfield 2019). Furthermore, this should also be reconsidered since antibiotic resistance is now a major global concern (Cerquetella et al. 2020).

The terms ‘’Inflammatory Bowel Disease (IBD)’’ and ‘’Chronic Enteropathy’’ should not be used interchangeably. The term "IBD" in veterinary medicine denotes a condition for which diet-based treatments and targeted treatment for dysbiosis have failed and whose cause is idiopathic. It is also necessary that inflammation has been proven on histopathological analysis (Dandrieux 2016; Hall and Day 2017).

2.1.1 – Prevalence

According to Jergens and Simpson (2012), IBD is the most common histopathological diagnosis in dogs and cats with chronic gastrointestinal clinical signs. However, these authors do not differentiate chronic enteropathy from IBD, encompassing everything in the term IBD.

Age of affected animals by chronic enteropathy varies according to the specific type of enteropathy. For instance, FRE is more common among young dogs and it's usually less severe (Allenspach et al. 2007). ARE is also more prevalent in young dogs with German Shepherds being predisposed (Batt et al. 1983; Johnston 1999; Hall 2011). On the contrary, dogs with IBD, tend to be middle-aged although intermittent signs might occur at an early age (Hall and Day 2017).

Dandrieux and Mansfield (2019) reviewed several studies to identify the true prevalence of the disease, although certain limitations were found. The fact that most of the studies reported general gastrointestinal signs, both acute and chronic of different etiologies, instead of the prevalence of chronic enteropathy itself is an example of those limitations. Despite this, it was possible to establish the prevalence of gastrointestinal disorders in general practice, which ranged from 1% (Marchesi et al. 2017) to 17.8% (O′Neill et al. 2014). Although the true prevalence of IBD is not known, it is suspected that it is over diagnosed, as clinicians do not always exclude all possible causes of intestinal inflammation before performing biopsy and histologic evaluation (Hall and Day 2017).

2.1.2 – Pathogenesis

Understanding the tolerance response to commensal and dietary antigens is necessary for knowing how an uncontrolled inflammatory reaction is generated. Antigen-presenting cells (APCs) continuously present antigens from the intestinal lumen to toll-like receptors (TLRs) on intestinal mucosal-associated lymphoid tissue (MALT) and on the basolateral surface of enterocytes. The TLRs are able to identify microbe-associated molecular patterns (MAMPs), which are molecules shared by all bacteria. When the antigens presented correspond to commensal organisms, naïve T cells differentiate into regulatory T cells (Treg and Th3) producing anti-inflammatory cytokines like interleukin (IL)-10 and transforming growth factor (TGF)-β (Hall and Day 2017). On the other hand, when pathogen-associated molecular patterns (PAMPs) are presented to these TLRs, the production of proinflammatory cytokines via NF-kappa-B is induced triggering the inflammatory cascade (Hall and Day 2017). In this scenario, naïve T cells differentiate into different types of T helper cells (Th1, Th2 or Th17) according to the invading pathogen (Hall and Day 2017).

The pathogenesis of chronic enteropathy (CE) is still not fully understood. However, similarly to Crohn's disease and Ulcerative Colitis in human medicine, it is currently

suspected that it results from the interaction of environmental factors, like dietary and microbiota antigens, and dysregulated immune responses in a genetically susceptible individual (Sartor 2006; Jergens and Heilmann 2022).

Overall, it can be claimed that CE arises from a loss of tolerance towards the autologous antigens. Immune tolerance consists in a balance between an intact mucosal barrier, an appropriately functioning mucosal immune system and an endogenous microflora population (German et al. 2003).

Although it is not known whether an alteration of the microbiota might be the cause or consequence of CE, it is known that it plays an important role in the development of the disease (Jergens and Heilmann 2022). The fact is that an abnormal inflammatory response occurs, either because there has been a change in the intestine microbial population (dysbiosis) or owing to abnormal host responses against the commensal microbiota, due to genetic variations (Jergens and Simpson 2012). This change in the microbiota has been demonstrated previously in molecular studies. In these studies, the small intestinal microbiota of dogs affected by CE presented elevated levels of Proteobacteria, particularly *Escherichia coli*-like, *Pseudomonas*, *Diaphorobacter* and *Acinetobacter* accompanied by reductions in *Firmicutes* and *Bacteroidetes* (Xenoulis et al. 2008; Suchodolski et al. 2010; Suchodolski et al. 2012; Honneffer 2014).

The role of genetics is evident in the predisposition of certain breeds to the disease. A study carried out in the United Kingdom (Kathrani et al. 2011) identified that the Weimaraner, the Rottweiler, the German Shepherd, the Border Collie and the Boxer were at higher risk of developing CE. Another evidence supporting the genetic involvement is the observation that certain forms of CE are only seen in certain breeds or pedigree lines, like the immunoproliferative enteropathy in Basenjis (MacLachlan et al. 1988), the histiocytic ulcerative colitis in Boxers (Hill and Sullivan 1978), the protein-losing enteropathy in Soft-Coated Wheaton Terriers (Vaden et al. 2000) and the diarrheal syndrome in Lundehunds (Flesjå and Yri 1977).

In any case, whether the origin remains in dysbiosis or genetic alterations, tolerance to the commensal microbiota is lost. Recognition of these antigens by TLRs leads to the production of cytokines, stimulating naïve T cells to become effector cells (Th1, Th2 or Th17) with the consequent production of more inflammatory cytokines (IL-17, IL-22, and TNF- α). These cytokines cause inflammation and epithelial cell damage, leading to disruption of the mucosal barrier, increasing its permeability. This perpetuates the cycle of antigen exposure and inflammation. All of these factors prevent Treg cells from regulating the situation, leading to the establishment of CE (Hall and Day 2017).

2.1.3 - Diagnosis

Chronic enteropathy has a wide range of clinical signs, but the most common ones are diarrhea and vomiting, which are frequently accompanied by weight loss and decreased appetite (Jergens and Simpson 2012). For this reason, the diagnosis of chronic enteropathy is difficult and requires ruling out other causes that mimic the disease's clinical signs (Jergens and Heilmann 2022).

CE is typically diagnosed by carefully combining information obtained from clinical signs, the animal's environment, history, physical examination findings, clinicopathologic tests, diagnostic imaging and the histological evaluation of intestinal samples (Simpson and Jergens 2011; Jergens and Heilmann 2022). All these parameters will allow the exclusion of extra-intestinal causes such as hypoadrenocorticism, hypercalcemia, exogenous pancreatic insufficiency, hepatic and renal diseases. Additionally, they allow ruling out parasitic and infectious diseases, as well as intestinal diseases of other etiologies like obstructions, tumors, and foreign bodies (Simpson and Jergens 2011; Dandrieux 2016).

2.1.3.1 - Hematology

Non-regenerative anemia associated with chronic inflammation or blood loss in the gastrointestinal tract may be present. Neutrophilia may also occasionally be present, with or without a left shift. Eosinophilia can also be detected, often associated with eosinophilic enteritis but not invariably since it is not pathognomonic of the disease. Thrombocytopenia and thrombocytosis are uncommon, with thrombocytopenia only appearing in less than 3% of cases. Thrombocytosis is a little more frequent and is sometimes associated with chronic gastrointestinal bleeding (Jergens and Simpson 2012; Hall and Day 2017).

2.1.3.2 - Serum Biochemistry and Vitamin Concentrations

Pathognomonic evidence for IBD is rarely provided by biochemical analysis results but these might help recognizing abnormalities in other organs that mimic CE clinical signs (Jergens and Simpson 2012; Hall and Day 2017). Despite that, hypocholesterolemia, hypocalcemia and hypomagnesemia are frequently reported. In cases of PLE, hypoalbuminemia and hypoglobulinemia are also common findings (Jergens and Simpson 2012; Hall and Day 2017). Numerous investigations have demonstrated that hypoalbuminemia is associated to a worse prognosis (Craven et al. 2004; Allenspach et al. 2007). Moreover, it has also been demonstrated that it is linked to several histological changes that arise in higher severity and that are less frequent in normoalbuminemic animals

(Wennogle et al. 2017). Furthermore, liver enzymes might be slightly elevated due to a reactive hepatopathy to the intestinal inflammation (Hall and Day 2017).

In what regards to vitamin concentrations, hypovitaminosis D may be detected (Hall and Day 2017). In a study conducted by Titmarsh et al. (2015) hypovitaminosis D was a strong predictor of death in dogs with CE. Therefore, this parameter might be associated with a worse prognosis. The intestinal malabsorption that occurs with inflammation can lead to low concentrations of folate and cobalamin which are absorbed at the proximal and distal ends of the small intestine, respectively (Hall and Day 2017). Both indicators could be low in the presence of generalized inflammation (Hall and Day 2017). A cobalamin concentration lower than 200 ng/L is associated with a negative outcome, thus, low serum cobalamin levels at the time of diagnosis may predict that the animal will be refractory to treatment (Allenspach et al. 2007). Similarly to what happens with hypoalbuminemia, hypocobalaminemia is also associated with histological alterations since it correlates with an increased number of intraepithelial lymphocytes in the ileal mucosa (Procoli et al. 2013). Although low serum cobalamin levels are associated with a worse prognosis, cobalamin supplementation is essential for successful treatment (Kather et al. 2020).

2.1.3.3 - Fecal Examination

Fecal examination is important to rule out parasitic causes like nematodes and protozoa and the techniques that are most used are usually direct wet mount and flotation (Jergens and Simpson 2012). However, cases of *Giardia* and *Cryptosporidium* are more easily detected through indirect-fluorescent antibody (IFA) tests (Hall and Day 2017). Animals with bloody stools, cytologic evidence of mucosal inflammation, fever or an inflammatory leukogram should undergo investigation to rule out the presence of pathogenic bacteria like *Salmonella* spp*., Campylobacter jejuni* and *Clostridium difficile* (Jergens and Simpson 2012; Hall and Day 2017). This investigation may be conducted through fecal culture or PCR of fecal material. However, fecal culture's value is often questionable while PCR appears to offer greater sensitivity as it does not require the presence of live organisms and facilitates speciation (Hall and Day 2017). Additionally, in a study conducted by Werner et al. (2021), it was found that fecal culture results exhibit notable interlaboratory variations, which is not observed in PCR. Nevertheless, in both these diagnostic methods, it is necessary to consider that many animals can be healthy and carriers of enteroinvasive bacteria, exhibiting subclinical carriage (Maciel et al. 2011; Goni et al. 2017; Hall and Day 2017; Werner et al. 2021). The significance of positive results should therefore be assessed in the context of the clinical presentation of each individual case.

Furthermore, it would also be expected that fecal Alpha1-Proteinase Inhibitor (α1-PI) concentration was elevated in IBD (Hall and Day 2017). This marker is a protein that is present in plasma, interstitial fluid and lymph. It has the same molecular weight as albumin, it's resistant to proteolytic degradation and it's usually only found in the intestinal lumen when one of the above mentioned fluids is lost. Therefore, it can be used as a marker for protein loss into the intestine (Murphy et al. 2003). Fecal α1-PI concentration proved to be useful in detecting early or less severe cases of CE before serum albumin concentrations decrease (Murphy et al. 2003). Besides, the highest concentrations of this marker were found in dogs with histologic changes compatible with IBD and lymphangiectasia (Murphy et al. 2003). For that reason, Murphy et al. (2003) suggested that, while treatment trials may be more acceptable in dogs with normal fecal α1-PI excretion, greater levels of this marker may signal the necessity for a gastrointestinal biopsy.

Another marker for intestinal inflammation is fecal calprotectin concentration (Hall and Day 2017). Calprotectin is a Ca^{2+}-binding protein that is associated with acute and chronic inflammation and also with malignant transformation (Heilmann and Allenspach 2017; Heilmann et al. 2018). Several studies have shown that this protein is correlated with clinical disease activity (Grellet et al. 2013; Heilmann et al. 2014; Otoni et al. 2018) and Heilmann et al. (2018) concluded that this marker has great utility in determining the disease classification and predicting the response to treatment.

2.1.3.4 - Diagnostic Imaging

The two most used diagnostic imaging tests are abdominal radiology and ultrasonography. However, chronic enteropathies generally do not show significant abnormalities on plain radiographs (Hall and Day 2017).

Abdominal Ultrasonography surpasses radiology in sensitivity and specificity in detecting intussusceptions, focal masses, foreign bodies, thickening of the mucosa and lymphadenopathy (Hall and Day 2017). Moreover, ultrasonography also assists in the detection of obstructions, loss of layering, hypoechoic and hyperechoic appearances like striations and speckles as well as non-gastrointestinal disease (liver, spleen, pancreas, lymph nodes, adrenals, masses and effusions) (Gaschen et al. 2008; Simpson and Jergens 2011).

Additionally, the use of fine needle aspiration during an ultrasonographic examination enables the collection of samples for cytological investigation from enlarged lymph nodes and localized wall thickening (Jergens and Simpson 2012).

According to Gaschen et al. (2008), wall thickness is not a very reliable indicator. In fact, echogenicity of the intestinal mucosa and secondary abnormalities of the intestine and

contiguous organs, such as free fluid, pancreatic edema and intestinal dilation, are the most significant variables to allow differentiation between the various types of CE (Gaschen et al. 2008). In fact, wall thickness might be insensitive and non-specific for both detection and differentiation of the type of CE and may lead to false negative results as it is not pathognomonic of disease (Gaschen et al. 2008; Hall and Day 2017).

Additionally, in that same study carried out by Gaschen et al. (2008) the ultrasonography score was associated with clinical activity at the time of presentation but not after treatment (4 weeks and 7 to 10 weeks after treatment). This suggests that despite improvement of clinical activity, the mucosa might require a longer time to heal, causing the ultrasonographic alterations not to correspond to the clinical score after treatment (Gaschen et al. 2008).

2.1.3.5 - Endoscopy and Mucosal Biopsy

In order to demonstrate intestinal inflammation, which is necessary for the diagnosis of IBD, intestinal biopsies are required (Washabau et al. 2010; Hall and Day 2017). Such biopsies can be obtained via flexible endoscopy, laparoscopy or surgery (Washabau et al. 2010), with endoscopy being considered the safest method (Hall and Day 2017).

Endoscopy enables the operator to observe and locate mucosal alterations that the surgeon's serosal approach would not allow to detect, thus facilitating direct biopsy of these lesions. In specific cases like ulceration, erosion and lymphangiectasia, this approach also enables the diagnosis of such lesions without the requirement for tissue biopsy. Furthermore, with this technique, many tissue biopsies can be taken with little danger of perforation and peritonitis. In general, this consists of a quicker, less stressful, less invasive and less expensive method than surgery (Washabau et al. 2010). On the other hand, with this biopsy method, samples are superficial and can be small and crushed, making it easy to obtain inadequate tissue samples that might prevent the establishment of a diagnosis (Washabau et al. 2010; Hall and Day 2017).

The GI Standardization Group developed report forms (Washabau et al. 2010) in order to guarantee a systematic, rigorous and complete examination of the gastrointestinal tract when performing an endoscopy. These forms include patient identification, date, reason for procedure, specific equipment used, complications encountered during the exam, extent of examination and images generated. Additionally, a comprehensive list of all the lesions that may be found in the different anatomical zones throughout the examination is provided, allowing clinicians to classify them on a scale of 0 to 3. Final recommendations are also included.

Mucosal friability, increased granularity and mucosal erosions are the most commonly reported endoscopic abnormalities encountered in dogs (Jergens et al. 1992; Allenspach et al. 2007; García-Sancho et al. 2007).

Allenspach et al. (2007) did not find any correlation between endoscopic scores and CIBDAI or histologic scores. García-Sancho et al. (2007) reached the same conclusions in an investigation where endoscopic lesions from dogs with lymphocytic-plasmacytic enteritis did not entirely disappear despite the post-treatment clinical improvements seen. In certain studies, the small intestine's endoscopic appearance correlates better with outcome than its histopathologic appearance (Allenspach et al. 2007; Mandigers et al. 2010). According to Hall and Day (2017), ileal biopsies appear to give a higher diagnostic yield. However, samples from that intestinal segment cannot be obtained by gastroduodenoscopy, therefore, an ileoscopy via colonic intubation might be necessary.

2.1.3.6 - Histopathology

The gold-standard for diagnosis of gastrointestinal disease is histopathology of gastric and intestinal biopsies (Hall and Day 2017). However, interpreting histological samples still has several limitations, including technical constraints, such as specimen size and processing artifacts, and excessive subjectivity due to interobserver variability (Willard et al. 2002). For this reason, Washabau et al. (2010) considered the following aspects important for the pathologist to take into account during the diagnostic process: the size and quality of the samples, the nature of the inflammatory response, the severity of the inflammatory response and to assess when an inflammatory response might be a precursor to lymphoid neoplasia. It is suggested that, given the limitations of microscopical examination of hematoxylin and eosin staining, immunohistochemistry might provide a more precise method to assess inflammation (German et al. 2001).

In order to achieve a less variable method for analyzing histological samples, the World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization Group developed standards for the diagnosis and treatment of gastrointestinal disease in the dog and cat. The group presents a method where a systematic approach to samples is applied and where the major findings are documented in a tick-box format. Guidelines with visual and textual description of the major inflammatory changes in the stomach, duodenum and colon are provided and, by the end of the analysis, the inflammatory changes are classified as mild, moderate or marked (Day et al. 2008).

The WSAVA Gastrointestinal Standardization Group identified the four most sampled regions of the gastrointestinal mucosa (gastric body, gastric antrum, duodenum and colon)

and then defined morphologic and inflammatory criteria to evaluate each of the regions (Annex 2) (Day et al. 2008).

According to the type of inflammatory cell that invades the intestinal mucosa, pathologists can deduce potential causes of the enteropathy. Infiltration with macrophages or neutrophiles might indicate the possibility of an infectious process and, in order to identify what agents might be present, the pathologist should use techniques such as culture, special stains and fluorescence in situ hybridization (FISH) (Jergens and Simpson 2012). The presence of eosinophils in intestinal biopsies may indicate parasitic infestation or dietary intolerance (Jergens and Simpson 2012). Identification of increased intraepithelial lymphocytes may assist in the diagnosis of chronic mucosal inflammation associated with IBD (Uzal et al. 2015).

The two most common forms of IBD in dogs are lymphoplasmocytic and eosinophilic enteritis, both of which are characterized by the infiltration of the corresponding cell type into the mucosa (German et al. 2003).

Overall, there is no strong association between clinical signs and histopathological findings in dogs with IBD (Washabau et al. 2010). However, there is proof that dogs with moderate-to-severe IBD with an increase in C-reactive protein (CRP) are more likely to have severe histologic lesions when compared to dogs with only mild clinical signs (Jergens et al. 2003). More recently, Allenspach et al. (2019) demonstrated that the methodical WSAVA guidelines for histopathological evaluation of samples improved the correlation between histopathological findings and scoring indexes.

In addition, it is also suggested that, in the colon, the loss of goblet cells and mucus may be associated with the severity of lymphoplasmocytic colitis (van der Gaag 1988; Roth et al. 1990; Mansfield et al. 2009). Willard et al. (2010) also demonstrated a connection between lacteal dilation in histological changes and the presence of hypoalbuminemia in biochemical analyses. The fact that there are so few associations with histopathological findings further supports the idea that, even though endoscopic biopsy is crucial for documenting inflammation, it cannot be used alone to make a diagnosis or determine a patient's prognosis (Washabau et al. 2010).

2.1.3.7 – Scoring Indexes for Disease Activity

Clinical activity indices enable measurement of IBD severity and help clinicians and researchers to assess the response to treatment and prognosis (Hall and Day 2017).

The Canine IBD Activity Index (CIBDAI) is a numerical scoring system consisting of 6 parameters (attitude/activity, appetite, vomiting, stool consistency, stool frequency and weight loss) each of which is assigned a value from 0 to 3 depending on severity. The values

assigned to each of the 6 variables are summed and the final value is obtained. If the final value is between 0 and 3, the disease is classified as ‘’Clinically Insignificant Disease’’, if the value is between 4 and 5 the disease is classified as ‘’Mild IBD’’, if the value is between 6 and 8 the disease is classified as ‘’Moderate IBD’’ and finally, for values above 9, the disease is classified as ‘’Severe IBD’’ (Jergens et al. 2003).

A study carried out by Jergens et al. (2003) demonstrates that CIBDAI correlates with histological severity and serum concentrations of acute phase proteins, especially CRP. In effectively treated dogs, both the CIBDAI and the CRP decrease, indicating that CRP is a suitable laboratory indicator of the therapeutic effect in these cases (Jergens et al. 2003).

The Canine Chronic Enteropathy Clinical Activity Index (CCECAI) scoring adds the assessment of serum albumin concentrations, ascites, peripheral edema and pruritus to the CIBDAI scoring system (Allenspach et al. 2007). In a study carried out by Allenspach et al. (2007), it was shown to be a much more powerful tool for accurately predicting a negative outcome.

2.1.3.8 – Other Diagnostic Tools

In addition to the aforementioned diagnostic techniques, there are additional approaches that might occasionally be required due to histopathology’s limitations. There are also promising approaches for the future that still need further investigation.

The dysbiosis index consists on a quantitative PCR of several key bacteria species that are usually altered in CE and is a very useful tool to assess dysbiosis (Allenspach and Mochel 2022). For that reason, it can also be used to verify treatment success (Allenspach and Mochel 2022). *Faecalibacterium, Fusobacterium, Clostridium hiranonis, Blautia and Turicbacter* are usually decreased, and *Streptococcus* and *Escherichia Coli* are typically increased in a dog with dysbiosis (AlShawaqfeh et al. 2017; Suchodolski 2022). A dysbiosis index above 2 indicates dysbiosis, with higher values indicating greater deviation from normal, while values between 0 and 2 suggest minor shifts in the microbiome (Suchodolski 2022).

Serum antibodies known as perinuclear anti-neutrophilic cytoplasmic antibodies (pANCA) are present in both humans and canines with a range of autoimmune diseases. These antibodies are directed against the myeloperoxidase found in neutrophils and macrophages and have been proven to cross-react with PAMPs. Consequently, chronic stimulation by PAMPs or molecules that mimic them in CE, leads to increased pANCA in circulation, thus making these antibodies useful markers in the early detection of the disease (Allenspach and Mochel 2022).

As previously mentioned, CE seems to have a genetic component, with several mutations associated with development of disease. Therefore, it is expected that soon, knowledge of the various genetic markers of the disease will be incorporated into commercially available tests (Allenspach and Mochel 2022).

MicroRNA and antibody measurements appear to be promising as well. In human medicine, the downregulation of certain microRNAs is used as a biomarker of the disease, however, additional research is still required in veterinary medicine (Allenspach and Mochel 2022). Antibodies against transglutaminase and gliadin also need further investigation but have already shown promising results in a study carried out by Vincenzetti et al. (2006). Growing interest is also emerging in the detection of antibodies against specific bacterial structures that usually increase in a situation of dysbiosis. Therefore, the detection of serum antibodies against *Escherichia coli* outer membrane porin C (OmpC) and flagellin is an interesting technique that also requires further studies (Allenspach and Mochel 2022).

2.1.4 – Treatment

Whenever possible, it is advised to use a staged therapeutic approach in the clinical management of chronic diarrhea. However, in some situations, clinical signs and inflammation are severe and prompt immunosuppressive treatment may be required (Hall and Day 2017). Treatment should be sequential and begin with empirical dewormer, which usually consists of a 5-day course of 50 mg/kg of febendazole (Simpson and Jergens 2011). Deworming is followed by a dietary trial as further detailed. While in the past, antibacterials were the further therapeutic step, nowadays, due to major global concerns regarding the long-term effects and appropriate antimicrobial stewardship, other options to modulate the microbiome have been studied (Cerquetella et al. 2020). These options include the use of prebiotics, probiotics or synbiotics (Rossi et al. 2014; White et al. 2017; Rossi et al. 2018) as well as fecal microbiota transplantation (Cerquetella et al. 2020). Finally, immunosuppressants are used in case animals do not respond or only show a partial response to the administered medication (Hall and Day 2017).

2.1.4.1 - Dietary Trial

FRE includes both dietary allergy triggered by an aberrant immune response and food intolerance through non-immunological mechanisms (Kennis 2006; Hall and Day 2017). The theory behind dietary therapy for FRE is that limiting exposure to antigens that are known to elicit sensitivity might decrease exacerbated host reactions and intestinal inflammation (Marchesi et al. 2017; Tolbert et al. 2022). In dogs with this type of chronic

enteropathy, the response to dietary change appears within one to two weeks (Makielski et al. 2019). If there is a positive response, the diet should be continued. However, in order to confirm the diagnosis, rechallenge with the original diet may be done (Hall and Day 2017). If after two dietary attempts there are no successful results, adjuvant treatments should also be administered (Makielski et al. 2019).

Traditionally, an intact protein elimination diet has been recommended (Jergens and Simpson 2012). However, more recently, hydrolyzed diets have also been used in FRE therapy with great success even in patients that initially did not respond to intact protein elimination diets (Hall and Day 2017). The effectiveness of a hydrolyzed diet over a novel antigen diet remains unclear (Tolbert et al. 2022), as studies show comparable outcomes and no significant difference in clinical response between the two (Allenspach et al. 2016; Dandrieux 2016). However, a research led by Marchesi et al. (2017) showed that hydrolyzed protein diets seemed to be more effective than novel protein diets in reducing relapses. Similarly, in a study by Mandigers et al. (2010) dogs initially showed similar responses to dietary changes with hydrolyzed and highly digestible but non-exclusion diets. However, the long-term remission rate was significantly better in dogs fed the hydrolyzed diet. Although the hydrolyzed diet is reflected in an improvement in clinical signs, histological changes do not complement clinical improvements (Hall and Day 2017). This suggests that either the hydrolyzed diet is so digestible that it is tolerated by the immune system or that the disease goes into remission but the histological improvement takes longer than the clinical improvement (Hall and Day 2017).

The main characteristics of a suitable diet for this disease focus on the presence of a novel intact protein, such as whitefish, duck, venison or soy, or an hydrolyzed protein source (Hall and Day 2017; Tolbert et al. 2022). A highly digestible carbohydrate source (e.g. well-cooked rice) should also be part of this diet and, because it is so important to follow a gluten-free diet, foods like potatoes, corn starch and tapioca are also excellent sources of carbohydrates (Jergens 1999; Jergens and Simpson 2012; Hall and Day 2017). It is important to note that, while less prevalent than protein-induced allergies, carbohydrates can often be the allergens responsible for eliciting allergic reactions to food (Verlinden et al. 2006; Bhagat et al. 2017). Therefore, in an exclusion diet, it is important to introduce both a novel protein and a carbohydrate to which the animal has not been regularly exposed as well (Paterson 1995; Johansen et al. 2017). Lastly, the diet should also be low in lactose and fat, nutritionally balanced and highly palatable (Jergens 1999; Jergens and Simpson 2012).

If serum concentrations are below normal, folate and cobalamin supplementation is advised (Hall and Day 2017; Tolbert et al. 2022). While it is still debatable whether cobalamin should be preferably administered PO or parenterally (Hall and Day 2017), a recent study

demonstrated that the difference in cobalamin concentrations before and after supplementation was consistently higher in orally supplemented animals (Chang et al. 2022).

2.1.4.2 - Antibiotic Trial

Antibiotic-responsive enteropathy has been historically considered as one of the variations of chronic enteropathy (Dandrieux 2016). However, some authors have suggested replacing the term ‘’ARE’’ to ‘’idiopathic intestinal dysbiosis’’ (Jergens and Heilmann 2022).

The mechanism by which antibiotics result in the improvement of clinical signs in these animals is unclear. They are used to counter the effects of microbial dysbiosis by modifying the intestinal flora through antimicrobial effects. Additionally, their immunomodulatory properties contribute to the modulation of the immune system (Dandrieux 2016; Hall and Day 2017).

Metronidazole (10-15 mg/kg given orally every 12 hours), tylosin (20 mg/kg given orally every 8/12 hours) and oxytetracycline (10-20 mg/kg given orally every 8 hours) were presented by Hall and Day (2017) as suitable treatment options for this purpose.

The indiscriminate use of antibiotics is not recommended and this therapy should only be reserved for patients in which other empirical treatments have been exhausted (Cerquetella et al. 2020). It is important to emphasize this because, nowadays, the intestinal microbiota is considered the dynamic reservoir of antibiotic resistance due to the indiscriminate use of such therapies (Rizzatti et al. 2018). Alternatives for the management of intestinal dysbiosis will be mentioned in the following section.

2.1.4.3 - Prebiotics, Probiotics, Synbiotics and Fecal Microbiota Transplantation

It is now beyond dispute that the intestinal microbiota and gastrointestinal homeostasis are associated (Minamoto et al. 2015; Dandrieux and Mansfield 2019). For that reason and given the downsides of the indiscriminate use of antibiotics, alternative therapies to modify microbiota are receiving considerable interest and clinical support (Cerquetella et al. 2020). Therefore, prebiotics, probiotics and synbiotics are alternatives that decrease mucosal inflammation, counterbalancing dysbiosis and providing a more advantageous risk-benefit profile for patients (Hansen and Sartor 2015).

Probiotics are live microorganisms said to provide health advantages to the host when taken in sufficient amounts, whereas prebiotics are defined as nondigestible food ingredients that stimulate the growth and activity of pre-existing colonic bacteria (FAO/WHO

2001). Synbiotics are a combination of a prebiotic and a probiotic (Jergens and Heilmann 2022). As demonstrated by several research (Rossi et al. 2014; White et al. 2017) multi-strain probiotics increase the expression of tight junction proteins, improving the intestinal barrier integrity, thus decreasing intestinal inflammation. Moreover, probiotics may also influence on the modulation of the immune response as several studies have shown an increase in regulatory T cell markers after their administration (Di Giacinto et al. 2005; Sauter et al. 2005; Rossi et al. 2014).

Another promising modality to assess this issue is fecal microbiota transplantation (Cerquetella et al. 2020). Fecal microbiota transplantation involves the transfer of intestinal content from a healthy donor to a diseased animal, to improve its intestinal health and decrease diarrhea (Chaitman et al. 2020). This procedure is a longstanding treatment in human medicine and is now being applied to canine patients by the veterinary community (Tuniyazi et al. 2022). Despite uncertainty about the exact mechanisms of fecal microbiota transplantation, four widely discussed hypothesis include niche exclusion, increased competition for nutrients, antimicrobial production and increased production of secondary bile acids (Tuniyazi et al. 2022). In human medicine, fecal donors are subjected to very rigorous screening procedures, while in veterinary medicine screening protocols vary among published studies (Chaitman and Gaschen 2021). Chaitman and Gaschen (2021) recommend a comprehensive evaluation of history, physical examination, and various laboratory analyses to determine suitability for canine fecal donation, with a key parameter being a fecal dysbiosis index less than 0. Moreover, the route of administration may not be as important as initially thought, as several methods of administration have shown similar efficacies (Tuniyazi et al. 2022). The amount of transplanted donor feces into the recipient is adapted from human medicine, typically utilizing 20 to 100 grams in each procedure (Cammarota et al. 2017; Chaitman and Gaschen 2021). Regarding frequency of administration, available data suggests that dogs with acute diarrhea often have positive responses to a single fecal microbiota transplantation (Chaitman et al. 2020). On the other hand, dogs with chronic intestinal diseases associated with dysbiosis may require multiple fecal microbiota transplantations (Chaitman and Gaschen 2021). The effects of this procedure in chronic enteropathies are not very well documented and further studies are required to asses which dogs could benefit from it (Chaitman and Gaschen 2021).

2.1.4.4 - Immunosuppressants

Immunosuppressant-responsive enteropathy (IRE) is an idiopathic intestinal inflammation resistant to therapy with diet and targeted treatment for dysbiosis. The

diagnosis is only confirmed by histopathology and positive response to immunosuppressant therapy (Dandrieux 2016; Dandrieux and Mansfield 2019; Benvenuti et al. 2021).

Glucocorticoids are the most used immunosuppressive drugs, being prednisone, prednisolone and methylprednisolone the most commonly prescribed (Hall and Day 2017). Budesonide is also an option, although more often used in cats (Hall and Day 2017). In some instances, animals might be unresponsive to corticosteroid induction, the disease might recur when the dose is reduced or there may be adverse effects associated to glucocorticoids (Craven et al. 2004; Simpson and Jergens 2011; Hall and Day 2017). In these cases, a second immunosuppressive drug can be considered, either alone or in combination with glucocorticoids. The most commonly used ones are azathioprine, chlorambucil, cyclophosphamide and cyclosporine (Hall and Day 2017).

The initial dose of prednisolone should be between 1 and 2 mg/kg, administered orally every twelve to twenty-four hours (as individually judged) (Jergens and Heilmann 2022). After about 2 to 4 weeks, this dose is gradually reduced over the succeeding weeks or months (Jergens and Heilmann 2022). Therapy with budesonide starts with 1, 2 or 3 mg per dog according to its size (Hall and Day 2017). Despite being an effective option, the study carried out by Dye et al. (2013) demonstrated that budesonide has no benefits compared to prednisolone.

Regarding cytotoxic drugs, despite the association of prednisolone and chlorambucil being indicated as very effective by various authors (Dandrieux et al. 2013; Hall and Day 2017), there has been a growing use of the prednisolone and cyclosporine combination, which is now more commonly observed in clinical practice, despite the lack of supporting literature. Several studies (Allenspach et al. 2006; Allenspach et al. 2007; Dandrieux 2016) have pointed to the effectiveness of cyclosporine in the treatment of IBD in dogs that did not respond to initial treatment with corticosteroids, it is therefore an excellent treatment option, although more expensive. If using azathioprine, regular complete blood count monitoring is recommended due to the drug's potential for myelosuppression (Hall and Day 2017). Cyclophosphamide is rarely used (Hall and Day 2017).

2.1.5 - Prognosis

According to cellular infiltration type or severity, chronic enteropathies may have different prognosis. The more severe the disease, the worse is the prognosis (Hall and Day 2017). Dogs with FRE have less severe clinical signs than other types of enteropathies (Allenspach et al. 2007). It can be stated that animals that respond quickly to a treatment with dietary trial alone are more likely to have a better prognosis (Allenspach et al. 2007; Dandrieux and Mansfield 2019). On the other hand, dogs that require steroid treatment are

usually older than FRE dogs and have more severe disease (Allenspach et al. 2007). This group is more likely to be euthanized since there is a higher chance of them being refractory to treatment (Allenspach et al. 2007). In fact, there is growing concern for dogs with ARE and IRE as, on the long term, they might not respond to therapy to the same degree as they did when first diagnosed (Dandrieux and Mansfield 2019).

As mentioned, the type of infiltrate also affects the prognosis. Simpson and Jergens (2011) claim that animals with eosinophilic enteritis usually have a good prognosis and continuous therapy with immunosuppressants is rarely required. As for animals with lymphangiectasia and crypt cysts/abscesses the prognosis is guarded (Simpson and Jergens 2011). Finally, the authors also affirmed that animals with idiopathic granulomatous or neutrophilic enteropathy will have a poor prognosis if the underlying cause is not identified (Simpson and Jergens 2011).

Furthermore, hypoalbuminemia has been identified as a poor prognostic indicator in numerous investigations (Craven et al. 2004; Allenspach et al. 2007; Benvenuti et al. 2021). In addition to this parameter, a decrease in body condition score (Benvenuti et al. 2021), a low serum cobalamin concentration (Allenspach et al. 2007), concurrent pancreatic disease indicated by high pancreatic lipase immunoreactivity (Hall and Day 2017) and hypovitaminosis D (Titmarsh et al. 2015) are also considered poor prognostic factors. Allenspach et al. (2007) also demonstrated that severe mucosal lesions in the duodenum were associated with negative outcome. Therefore, the identification of this kind of lesions on the duodenum of a dog with CE during an endoscopic procedure might justify the implementation of a more aggressive treatment early in the disease.

2.2 - Canine Leishmaniosis

2.2.1 - Etiology, Life Cycle and Epidemiology

Leishmaniosis is a group of infectious diseases caused by the protozoan of the genus *Leishmania* from the Kinetoplasta class and Trypanosomatidae family (Baneth, Petersen, et al. 2022). In Portugal, *Leishmania infantum* is the main agent responsible for the disease in dogs and in humans, with dogs being the reservoir host (Baneth, Petersen, et al. 2022). This species is a diphasic parasite given that it has a promastigote and an amastigote form. The promastigotes are mostly found in the natural vectors of this protozoan, the phlebotomine sand flies, which have two main genus: the *Phlebotomus* in the Old World (Europe, Asia and Africa) and the *Lutzomyia* in the New World (America) (Baneth, Petersen, et al. 2022; Morales-Yuste et al. 2022). Amastigotes are found in macrophages of vertebrate hosts where they multiply by binary fission, leading to the rupture of these phagocytic cells, infecting new ones. When a sand fly ingests blood from an infected dog, the amastigotes are released from the host cells in the sand fly's gut and transform into the flagellated promastigote form, starting their replication. When in an appropriate vector, this replication results in a detachment from the midgut epithelium and in an anterior migration to the foregut and mouthparts of the *Phlebotomus*. A new dog gets infected when the saliva from a female sand fly is injected in the skin of a vertebrate host. After this inoculation, the promastigotes lose their flagella taking the form of amastigotes again (Baneth, Petersen, et al. 2022; Morales-Yuste et al. 2022). In addition to sand fly bites, it has also been confirmed that dogs can become infected by venereal and vertical transmission, through blood transfusions, during fights or by using the same needle during veterinary procedures (Lappin 2020). Other means of transmission need further investigation, in particular, dog-to-dog transmission and transmission by the *Rhipicephalus sanguineus* tick (Lappin 2020; Baneth, Petersen, et al. 2022).

The geographic distribution of *Leishmania* focuses primarily in the Mediterranean Basin, in North Africa, South America and Western Asia (Morales-Yuste et al. 2022). According to seroprevalence studies from Spain, France, Italy and Portugal, it is estimated that 2,5 million dogs are infected with *Leishmania infantum* in these countries, whereas in America, there are very high infection rates in some areas of Brazil and Venezuela (Baneth, Petersen, et al. 2022).

In Portugal, leishmaniosis is an endemic disease and the number of cases has been increasing in the last few years (Campino and Maia 2010). According to a recent study (Almeida et al. 2022), the seroprevalence of leishmaniosis in Portugal has almost doubled over the last 10 years, rising from 6.3% in 2009 to 12.5% in 2021 (Almeida et al. 2022).

According to the same study, the two areas with the highest seroprevalence for leishmaniosis in the country are Castelo Branco and Portalegre, although Bragança, Guarda, Coimbra, Setúbal and Faro also presented high seroprevalence levels.

2.2.2 – Immune Response, Pathophysiology and Clinical Signs

As previously described, when a female sand fly bites a vertebrate host, *Leishmania* promastigotes are injected into its dermis. These promastigotes are then phagocytized by phagocytic cells such as neutrophils, dendritic cells and macrophages which may either be resident or recruited to the bite site (Hosein et al. 2017). The promastigotes shift into amastigotes within these cells and multiply by binary division, causing the host cells to enlarge and rupture. Thereafter, amastigotes infect other host cells and disseminate, firstly, via lymphatic circulation to the draining lymph node and secondly, to the hemolymphatic organs including the spleen, bone marrow, liver and other dermal areas (Baneth, Petersen, et al. 2022).

In classical canine leishmaniosis, the most common clinical signs include skin lesions, lymphadenomegaly, weight loss, muscular atrophy, splenomegaly, exercise intolerance, lethargy, hyporexia, onychogryphosis and ocular lesions. Other clinical manifestations include epistaxis, gastrointestinal disease, lameness, polyarthritis, polyuria and polydipsia and ascites (Ciaramella et al. 1997; Koutinas et al. 1999).

Not all dogs exposed to *Leishmania infantum* develop clinical manifestations. Therefore, dogs can be susceptible to disease, presenting clinical signs and developing severe illness, or resistant. Dogs resistant to *Leishmania* range from 10% to more than 50% and either do not develop disease or the disease resolves spontaneously (Koutinas et al. 1999; Solano-Gallego et al. 2009).

Leishmania infections typically induce a combined immune response characterized by the presence of both Th1 (cellular response) and Th2 (humoral response) cytokines, maintaining a delicate balance that, when broken, determines the outcome of either disease elimination or progression, respectively (Morales-Yuste et al. 2022).

Resistance to disease is associated with a driven Th1 immune response with production of IFN-γ, TNF-α and IL-2 (Barbiéri 2006; Hosein et al. 2017). The principal mechanism for protection against leishmaniosis remains on the activation of macrophages by IFN-γ and TNF-α. Once activated, macrophages prevent intracellular parasite replication and disease development by killing intracellular amastigotes via nitric oxide pathway (Hosein et al. 2017; Toepp and Petersen 2020). Th17 cells have also been recently proved to be involved in the protective immunity for leishmaniosis by releasing IL-17 which, consequently,

recruits neutrophils and macrophages to the site of infection (Toepp and Petersen 2020; Baneth, Petersen, et al. 2022).

This subclinical state is characterized by detection of parasites via quantitative PCR from blood samples, low antibody titers on ELISA and a strong delayed-type hypersensitivity response in the leishmanin intradermal skin test (Toepp and Petersen 2020).

On the other hand, IL10, IL13, IL4, and TGF- β lead to a Th2 immune response in which immunoglobulins are vastly synthesized, the macrophages are inactivated and the parasites can thrive (Morales-Yuste et al. 2022). The hyperproduction of immunoglobulins results in the formation and deposition of immune complexes in different locations. This causes polyarthritis, uveitis and glomerulonephritis, which is the primary reason for leishmaniosis-related deaths. Additionally, it can result in vasculitis, activating the complement cascade and leading to tissue necrosis and thus, dermal, visceral, ocular and renal lesions (Hosein et al. 2017; Baneth, Petersen, et al. 2022).

From a diagnostic perspective, at this stage of the disease, similarly to what was verified in the subclinical state, parasites are detectable via quantitative PCR of blood samples. However, contrary to the subclinical state, at this stage of disease, animals present high serologic titers (Toepp and Petersen 2020).

The progression of the disease depends on different factors, namely breed, age, gender, proportion of time spent outdoors, immunosuppression, concomitant disease and nutritional status (Solano-Gallego et al. 2009; Hosein et al. 2017). For instance, mongrel dogs or autochthonous breeds from endemic areas are provided with different tiers of resistance (Solano-Gallego et al. 2000). On the other hand, Rottweilers, Boxers, German Shepherds and Foxhounds have been shown to have genetic susceptibility to the disease (Ciaramella et al. 1997; Duprey et al. 2006; Miranda et al. 2008).

2.2.3 - Intestinal Manifestations of Leishmaniosis

In most cases of leishmaniosis, diarrhea is usually considered a secondary manifestation, often associated with kidney or liver disease (López et al. 2022). Nevertheless, chronic enteropathy, directly caused by parasite-induced inflammation, has been documented in many case reports and, occasionally, diarrhea is the sole clinical sign of the disease (Ferrer et al. 1991; Ruiz et al. 2015; Vila et al. 2016; Ayala et al. 2017; López et al. 2022). Intestinal inflammation arises when humoral immunity is hyperactivated. Gradually, the intestinal mucosa becomes infiltrated by lymphocytes, plasma cells and parasitized macrophages (González et al. 1990).

Several studies examined the intestine of animals positive for leishmaniosis, with specific focus on cases where diarrhea was a primary clinical sign. The evaluation

encompassed both macroscopic and microscopic analyses**.** The most reported macroscopic alterations were mild edema, mucosal multifocal erosions and mucosal hyperemia (González et al. 1990; Ferrer et al. 1991; Ruiz et al. 2015; Ayala et al. 2017; Ward et al. 2019). Less frequently, alterations such as increased friability and granularity of the mucosa and diffuse thickening of the mucosa have also been reported (González et al. 1990; Ayala et al. 2017).

In a study by Adamama-Moraitou et al. (2007), dogs with symptomatic leishmaniosis, despite lacking historical or clinical evidence of colitis, showed a positive correlation between macroscopic lesions observed during colonoscopy and colonic parasitism. The endoscopy performed revealed patches of hyperemic, edematous, irregular and mildly erosive colonic mucosa in 25,8% (8/31) of the animals.

Concerning microscopic changes, most of the cells that were found infiltrated at the level of the intestinal mucosa were macrophages, plasma cells and lymphocytes, compatible with a granulomatous form of inflammation with a lymphoplasmacytic component (Ruiz et al. 2015; Ayala et al. 2017; López et al. 2022). However, granulomatous inflammation sometimes presented without the associated lymphoplasmacytic component and the opposite was also true (López et al. 2022). In some cases, this type of inflammation was also accompanied by a neutrophilic infiltrate (González et al. 1990; Ferrer et al. 1991; Ward et al. 2019). In histological analysis of colon samples from his study, González et al. (1990) also identified some eosinophils.

Regarding the morphology of the gastrointestinal tract, lacteal dilation was identified in the duodenum and ileum of two dogs from López's et al. (2022) study, one of which presented signs of protein losing enteropathy. Ward et al. (2019) identified villous stunting in the ileum samples from his reported case and Adamama-Moraitou et al. (2007) described surface exudates, erosions, distortion of the epithelium, increased perivascular fibroplasia, crypts containing mucus and/or cellular debris in the lumen and crypt abscessation.

2.2.4 - Laboratory Findings

Numerous changes in the complete blood count and biochemical analyses can support the clinical suspicion of leishmaniosis.

According to numerous retrospective studies (Ciaramella et al. 1997; Koutinas et al. 1999; Freitas et al. 2012), a nonregenerative normocytic and normochromic anemia is frequently reported. Regarding white blood cells, the literature is slightly ambiguous since both leukopenia and leukocytosis can arise (Baneth, Petersen, et al. 2022). Concerning platelets, thrombocytopenia is often present along with thrombocytopathy (Ciaramella et al. 1997; Ciaramella et al. 2005; Baneth, Petersen, et al. 2022).

The most consistent serum biochemistry findings are hyperproteinemia with hyperglobulinemia and hypoalbuminemia, resulting in a decreased albumin/globulin ratio (Ciaramella et al. 1997; Koutinas et al. 1999; Freitas et al. 2012; Baneth, Petersen, et al. 2022). Clinicians must be on the lookout for leishmaniosis in dogs from endemic areas that have polyclonal gamma and beta hyperglobulinemia with no other obvious cause (Baneth, Petersen, et al. 2022). Acute phase proteins such as C-reactive protein and ferritin may also be elevated (Baneth, Petersen, et al. 2022).

As renal disease is one of the main causes of death associated with leishmaniosis, it is also often possible to find azotemia (Ciaramella et al. 1997; Koutinas et al. 1999; Freitas et al. 2012). Leishmaniosis related renal disease can range from mild proteinuria to nephrotic syndrome or an end stage renal disease (Solano-Gallego et al. 2011). Koutinas et al. (1999) reported 3 dogs with nephrotic syndrome presenting subcutaneous edema, hypercholesterolemia and severe hypoalbuminemia. Proteinuria is also a common finding in diseased dogs associated with glomerular impairment (Baneth, Petersen, et al. 2022).

A slight increase in liver enzymes is another potential finding (Ciaramella et al. 1997; Koutinas et al. 1999; Baneth, Petersen, et al. 2022).

2.2.5 - Diagnosis

A combined strategy that includes a clinicopathological assessment with specific laboratory tests is frequently needed for an accurate diagnosis of leishmaniosis. A suspicion of disease may be raised based on history, physical examination, complete blood count, biochemical analyses, urinalysis and serum electrophoresis. However, in order to reach a definitive diagnosis and identify the parasite or the patient's response to it (etiologic diagnosis), specific laboratory tests must be performed (Solano-Gallego et al. 2009; Paltrinieri et al. 2010; WOAH 2021). This tests are divided into two main categories: direct tests (cytologic and histologic evaluation, culture and PCR assay) and indirect tests that clarify the animal's immune response (serologic testing and evaluation of cellular immune response) (Paltrinieri et al. 2010; WOAH 2021).

2.2.5.1 - Direct tests

Direct tests include cytological and histological evaluation of samples for detection of *Leishmania* amastigotes within macrophages or extracellularly. More than identifying the agent itself, it may be possible to identify cytologic and histologic alterations consistent with leishmaniosis (Paltrinieri et al. 2010). If the parasite cannot be identified and only these changes are visible, immunohistochemistry methods can be applied to histological samples

(Bourdoiseau et al. 1997; Tafuri et al. 2004). Fine-needle aspiration and biopsies should be performed on sites that are injured or suggestive of being involved (e.g. synovial fluid in case of polyarthritis) (Paltrinieri et al. 2010). Regarding cytology, in the absence of signs that allow the agent to be located, amastigotes are most likely to be found in the spleen, bone marrow, lymph nodes and buffy coat from peripherally obtained blood, in descending order of diagnostic sensitivity (Mylonakis et al. 2005; Saridomichelakis et al. 2005).

Culture of *Leishmania* organisms is also considered a direct test. However, despite being 100% specific, it is less frequently employed for diagnosis due to its drawbacks, such as the delay in obtaining results (Maia and Campino 2008; Morales-Yuste et al. 2022).

Finally, PCR is a molecular method that has simplified the diagnosis of leishmaniosis as it identifies very low quantities of the protozoan DNA in biological samples (Paltrinieri et al. 2010). The PCR test should preferentially be performed on samples of bone marrow or lymph node, skin, conjunctiva, buffy coat or whole peripherally obtained blood, in decreasing order of sensitivity. Moreover, regardless of the biological material used, fresh or frozen samples or samples after fixation in 95% ethyl alcohol are always preferred over formalin-fixed paraffin-embedded ones, which give lower diagnostic yields (WOAH 2021). Real-time PCR is also a useful tool in the post-treatment period since, because of its quantitative nature, it allows the monitoring of *Leishmania* DNA load (Manna, Reale, Vitale, et al. 2008).

2.2.5.2 - Indirect tests

Serological assays or assessment of the cellular immune response can be employed as a means of indirect diagnosis. When natural infection occurs, the interval to seroconversion can oscillate between 1 to 22 months, with a mean of 5 months (Moreno and Alvar 2002). In order to detect these antibodies, using serological methods, several techniques can be applied. Indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and rapid immunochromatographic assay are the three most used techniques (Morales-Yuste et al. 2022). These tests usually have good sensitivity and specificity to detect *Leishmania*. However, these parameters vary with the type of antigen used in the test (Baneth, Petersen, et al. 2022). Whole parasite-extracts, like those used in IFAT technique, are very sensitive to detect subclinical and clinical infections but may provide a lower specificity (Maia and Campino 2008; Baneth, Petersen, et al. 2022). In contrast, assays that use recombinant protein antigens, like some ELISA variants, are very specific but may lack sensitivity for detection of clinically healthy dogs (Maia and Campino 2008; Baneth, Petersen, et al. 2022). Some authors consider IFAT the ‘’gold standard’’ of serologic diagnosis (Maia and Campino 2008; Morales-Yuste et al. 2022). However, when positive, the evaluation of fluorescence intensity is prone to subjective interpretation, which is

a disadvantage of the technique (Paltrinieri et al. 2010). On the other hand, in ELISA, when faced with a positive result, the colorimetric reaction that appears can be quantified via spectrophotometry preventing the technique from being subjective (Paltrinieri et al. 2010). This quantification is important as it allows the distinction between subclinically infected dogs, which usually have low antibody titers, and dogs with parasitic dissemination and disease, which usually have high antibody titers (Paltrinieri et al. 2010). Finally, as mentioned before, dogs that develop disease have depressed cellular response to *Leishmania*. Therefore, when cell-stimulation assays are applied, these animals show no response or little response to those tests (Cabral et al. 1993; Pinelli et al. 1994; Cardoso et al. 2007). The leishmanin skin test, the lymphocyte proliferation assay and the interferon-γ cytophatic effect inhibition bioassay evaluate the cellular response to *Leishmania* and would be useful for monitoring the evolution of the disease, response to treatment and prognosis, however, this tools are still unavailable for clinical practice (Maia and Campino 2008; Paltrinieri et al. 2010).

2.2.6 - Clinical staging

Following the diagnosis *of Leishmania infantum* infection, clinical staging is carried out to enable the patient's proper treatment and monitoring (Solano-Gallego et al. 2017).

Staging is carried out according to the stages defined by the LeishVet Group (Baneth, Bourdeau, et al. 2022). There are four clinical stages of disease and each dog is categorized based on clinical signs, laboratory alterations and quantitative serology.

Stage I corresponds to mild disease and in this stage only mild clinical signs are present (e.g. papular dermatitis or localized lymphadenomegaly), there are no clinicopathological abnormalities and antibody levels are negative or low positive.

Stage II corresponds to moderate disease and this stage encompasses cases with clinical signs such as diffuse or symmetrical cutaneous lesions, generalized lymphadenomegaly, loss of appetite and weight loss. Clinicopathological abnormalities like mild non-regenerative anemia, hypergammaglobulinemia and hypoalbuminemia might be present. This stage is subdivided into two substages: a) with a normal renal profile and b) with a urinary protein/creatinine (UPC) ratio in between 0,5 and 1. In this stage, the antibody levels can be low to high positive.

Stage III corresponds to severe disease and in this stage, dogs can show all the clinical signs of stages I and II and, in addition, lesions associated with deposition of immune-complexes may also appear (e.g. glomerulonephritis and uveitis). Clinical abnormalities from stage II may be present and chronic kidney disease (CKD) may also occur. It might be a CKD International Renal Interest Society (IRIS) stage I with UPC ratio

above 1 or a CKD IRIS stage II. In this stage, the antibody levels are medium to high positive.

Stage IV corresponds to very severe disease and in this stage, besides all the aforementioned clinical signs, dogs can have pulmonary thromboembolism, nephrotic syndrome and end stage renal disease. In addition to all the clinicopathological abnormalities listed in the previous stages, a CKD IRIS stage III, a CKD IRIS stage IV, nephrotic syndrome or marked proteinuria with a UPC ratio above 5 might be present. In this stage, the antibody levels are medium to high positive.

2.2.7 - Treatment

Therapy choices must be based on clinical staging (Solano-Gallego et al. 2011; Morales-Yuste et al. 2022). According to Solano-Gallego et al. (2011) stage I dogs can be monitored without treatment or require less prolonged treatment with one or two combined drugs. Whereas it is recommended that stage II and III dogs are treated with a combination of allopurinol and an antimonial or miltefosine (Solano-Gallego et al. 2011). Specific treatment for stage IV dogs should be instituted individually to avoid further kidney damage (Baneth, Bourdeau, et al. 2022; Morales-Yuste et al. 2022). Moreover, in the context of chronic kidney disease, the IRIS treatment guidelines for CKD should also be followed (Baneth, Bourdeau, et al. 2022).

The three drugs of first choice for the treatment of leishmaniosis are meglumine antimoniate, miltefosine and allopurinol (Reguera et al. 2016).

Meglumine antimoniate is a pentavalent antimonial. The therapeutic effects of this compound have been known for decades, yet it still remains the drug of choice for treatment of leishmaniosis. It can be administered as monotherapy or in combination with other drugs to amplify their antileishmanial effect and reduce the occurrence of relapses (Reguera et al. 2016). It must be administered subcutaneously, every 24 hours for four to eight weeks at a dose of 75 to 100 mg/kg (Baneth, Petersen, et al. 2022). Despite its known antileishmanial effect, meglumine antimoniate has several adverse effects, with the most reported ones being pain in the injection site associated with thrombophlebitis, local swelling and potential abscess formation (Slappendel and Teske 1997; Baneth, Petersen, et al. 2022). Gastrointestinal manifestations are also frequently reported (Slappendel and Teske 1997; Baneth and Shaw 2002) along with nephrotoxicity (Koutinas et al. 1999). Valladares et al. (1998) reported abnormally high values of total proteins specially in the gamma globulin fraction after starting of the treatment, although those values returned to normal within 3 months.

Miltefosine is an alkylphospholipid with a direct toxic effect on *Leishmania* parasites (Baneth, Petersen, et al. 2022). In some european countries, miltefosine is replacing meglumine antimoniate as a first choice treatment. However, and because miltefosine has no nephrotoxic effects, it is generally used after therapeutic failure with meglumine antimoniate, after relapse or due to renal disease (Bianciardi et al. 2009; Reguera et al. 2016). In addition to being toxic to *Leishmania* protozoa, some studies (Manna, Reale, Picillo, et al. 2008; Andrade et al. 2011) have also demonstrated the effect of miltefosine in potentiating the Th1 immune response, increasing IFN-γ, while suppressing the Th2 immune response, leading to reduced levels of IL-10 and IL-4. Miltefosine should be administered orally, every 24 hours for four weeks at a dose of 2 mg/kg (Baneth, Petersen, et al. 2022). The most reported side effects of miltefosine are gastrointestinal signs such as vomiting, anorexia and diarrhea, which can be reduced by administration with food (Baneth, Bourdeau, et al. 2022). Miltefosine has shown to be embryotoxic and fetotoxic in rats and rabbits and teratogenic in rats, therefore, and even though there is no data available regarding the development of this kind of toxicity in dogs, the use of this drug should be avoided during pregnancy (Reguera et al. 2016).

Allopurinol, a hypoxanthine analogous, is commonly combined with both meglumine antimoniate and miltefosine. It represents the most used compound against leishmaniosis in Europe, as a combined treatment (Mattin et al. 2014). In Portugal, it is the primary choice among general veterinary practitioners for treating stage II and III leishmaniosis when combined with meglumine antimoniate and for treating stage IV disease when combined with miltefosine (Monteiro et al. 2021). The "gold standard" for treating leishmaniosis, according to practitioners, is the combination of allopurinol and meglumine antimoniate (Reguera et al. 2016). Allopurinol should be administered orally in a dose of 10mg/kg, every twelve hours (Baneth, Petersen, et al. 2022). A reduction of parasite burden to an undetectable level and clinical improvement are achieved within four weeks of treatment with this compound (Baneth, Petersen, et al. 2022). The combination of allopurinol and meglumine antimoniate should be administered for four to eight weeks, period after which allopurinol should be continued as a monotherapy for at least another six to twelve months (Baneth, Petersen, et al. 2022). Allopurinol can be discontinued when a complete physical and clinicopathological recovery is confirmed by a thorough physical examination, complete blood count, biochemistry panel, urinalysis and when there is a marked decrease in antibody titers to a negative or borderline level on a quantitative serological assay (Solano-Gallego et al. 2009). Nephrotoxicity has not been reported. However, allopurinol induces the formation of xanthine crystals which can lead to urolithiasis (Torres et al. 2011; Baneth, Petersen, et al. 2022). Discontinuing allopurinol may be necessary if attempts to control allopurinol-induced

xanthinuria and uroliths through low-purine diets or reduced drug dosage are ineffective (Solano-Gallego et al. 2011).

Domperidone and dietary nucleotides can be considered for monotherapy in stage I leishmaniosis (Baneth, Bourdeau, et al. 2022). Domperidone stimulates the hypophysis to release prolactin which contributes to a Th1 immune response with production of IFN-γ , IL-2, IL-12 and TNF-α (Majumder et al. 2002). Dietary nucleotides also seem to enhance the Th1 immune response against *Leishmania*, thus modulating the immune system (Segarra 2021). It is suggested that they form part of the multimodal therapy for leishmaniosis, which must be individually adapted to each dog. For example, they could be considered as an alternative to allopurinol in patients with xanthinuria (Segarra 2021).

Other drugs could be administered for treatment of *Leishmania* infection. However, due to adverse effects associated to their use, they're not recommended. Pentamidine, amphotericine B and aminosidine are a few examples (Baneth, Petersen, et al. 2022).

Clinical cure is often achieved, especially with the meglumine antimoniate and allopurinol association. However, parasitological cure is rarely achieved and, for that reason, dogs can experience clinical relapse after the end of treatment (Solano-Gallego et al. 2009).

2.2.8 - Prognosis

The prognosis for each dog varies according to clinical and clinicopathological changes. For this reason, at the time of diagnosis, animals are grouped into different stages, which allows inferring both the most adequate therapy to institute and what can be expected from the treatment, meaning, the prognosis (Solano-Gallego et al. 2009). The prognosis can improve or worsen throughout the course of therapy as the animals stage may change. Bearing in mind the aforementioned clinical staging, the prognosis for animals with stage I disease is usually good. Stage II animals have a good to guarded prognosis. Conversely, stage III and stage IV animals have a guarded to poor and poor prognosis, respectively (Solano-Gallego et al. 2011).

2.2.9 - Prevention

For optimal prevention, a multimodal approach should be considered in endemic areas combining a topical insecticide and vaccination. Most topical insecticides are based on products containing synthetic pyrethroids like permethrin or deltamethrin (Solano-Gallego et al. 2011).

Several studies (Mencke et al. 2003; Miró et al. 2007; Otranto et al. 2007; Otranto et al. 2010) have demonstrated the efficacy of permethrin and imidacloprid spot on

formulations. This treatment provides a repellent effect against sand flies, thus preventing sandfly bites, for three weeks. Spot on formulations should be applied at least two days before traveling to an endemic area. The effectiveness of deltamethrin-impregnated collars has also been evaluated by several studies (Killick-Kendrick et al. 1997; Maroli et al. 2001; Gavgani et al. 2002; Foglia Manzillo et al. 2006) which have shown that these collars are effective in controlling sand fly feeding for five to six months, period after which they must be replaced. Deltamethrin-impregnated collars should be applied at least one to two weeks before traveling to an endemic area.

Nowadays, the only available vaccine in Europe against leishmaniosis is Letifend® (Leti Pharma laboratories) whose active principle consists of a recombinant protein, ‘’protein Q’’, containing five different antigens from *Leishmania infantum.* The first vaccination is given at 6 months of age and revaccinations are annual. Dogs should only be vaccinated if serologically negative (European Medicines Agency 2016; Baneth, Bourdeau, et al. 2022). The effectiveness of this vaccine is 72% and it reduces the risk of developing leishmaniosis after natural infection with *Leishmania infantum* (Fernández Cotrina et al. 2018).

In addition to vaccination and topical insecticides, there are a number of measures that can be taken to help prevent sandfly bites. Keeping pets indoors during the sandfly season from dusk to dawn, reducing the microhabitats favorable to sand flies near the house or in locations accessible for the pet and using environmental insecticides are a few examples (Alexander and Maroli 2003).

3 - Old Enemies, New Differentials: Canine Leishmaniosis As A Cause Of Refractory Enteritis

3.1 – Introduction

Canine leishmaniosis is endemic in Portugal with a notable rise in the number of cases in recent years (Campino and Maia 2010), alongside a twofold increase in its seroprevalence over the last decade (Almeida et al. 2022).

While the typical clinical signs of the disease (including dermatological changes, lymphadenomegaly, weight loss, muscular atrophy, splenomegaly, exercise intolerance, lethargy, hyporexia, onychogryphosis and ocular lesions) are well-documented (Ciaramella et al. 1997; Koutinas et al. 1999), some studies have drawn attention to cases where the sole manifestations were gastrointestinal.

In 1991, Ferrer et al. reported two clinical cases of dogs whose only clinical sign was chronic large bowel diarrhea. Subsequent immunoperoxidase staining from intestinal biopsies revealed their positivity to *Leishmania*. The dog whose owners opted for etiological treatment for *Leishmania* ceased diarrhea after 2 months of treatment and remained healthy after one year. Other authors described similar cases where the majority of the classic *Leishmania* clinical signs were absent and only those linked to chronic enteropathy were observed (Ruiz et al. 2015; Vila et al. 2016; Ayala et al. 2017). All animals in these studies presented diarrhea, weight loss and one of them also experienced episodes of vomiting. All of them were diagnosed with leishmaniosis after intestinal biopsies and revealed numerous macrophages with basophilic bodies within their cytoplasm. All animals had a complete resolution of clinical signs after etiologic treatment for *Leishmania* with meglumine antimoniate and allopurinol.

More recently, in 2022, a retrospective observational study was conducted by López et al. in four different veterinary referral hospitals, all settled in endemic areas for canine leishmaniosis in Spain. This study included 23 dogs with leishmaniosis whose main clinical sign was chronic diarrhea. Weight loss was the second most frequent clinical sign and all other signs reported were also gastrointestinal. Eight of the 23 canines that tested positive for *Leishmania* underwent endoscopy with gastrointestinal biopsies and *Leishmania* amastigotes were identified in all of them. All dogs that underwent specific treatment for leishmaniosis had a complete resolution of diarrhea.

Despite this, leishmaniosis is not included in the comprehensive workup to address chronic diarrhea (Willard 2017). In previous studies, it was shown that sometimes simpler and more cost-effective approaches could have identified the etiology of chronic diarrhea (Leib 2000; Bryan et al. 2019; Cerquetella et al. 2020; López et al. 2022). This raises

questions about whether a similar scenario might occur if veterinary practitioners conducted screening tests for *Leishmania* earlier in endemic regions.

Scarce information exists regarding the connection between chronic enteropathy and leishmaniosis in Portugal. Specifically, it remains unclear whether cases of leishmaniosis with exclusive gastrointestinal manifestations exist in Portugal, as, to the authors' knowledge, there are currently no studies reporting such occurrences.

This study aims to explore a possible connection between refractory chronic enteropathy (RCE) and leishmaniosis. We hypothesize that there may be up to 20 to 30 percent of dogs with RCE that are positive for the endemic protozoan *Leishmania infantum*.

3.2 – Materials and methods

A retrospective study was conducted including cases diagnosed with RCE, examined at the veterinary teaching hospital between January 2018 and October 2022.

The inclusion parameters dictated that dogs had to have a clinical history of RCE, and had undergone a gastroduodenoscopy or colonoscopy between the period of the study, with histopathology conducted at the Pathology lab, and available samples in the lab's archives. Dogs were eligible for the study if they had been followed up at the Internal Medicine Service of the Veterinary Teaching Hospital for gastrointestinal clinical signs lasting longer than 3 weeks. The diagnosis of chronic enteropathy was obtained at the Veterinary Teaching Hospital based on the outcomes of tests and clinical feedback. To be considered RCE, this diagnosis would have to be obtained after exclusion of extraintestinal causes (namely, hypoadrenocorticism, exogenous pancreatic insufficiency, liver and renal diseases, gastrointestinal parasites and infectious diseases) or intestinal causes other than chronic enteropathy (namely, obstructions, tumors and foreign bodies).

In order to classify chronic enteropathy as refractory, all animals must had a dietary modification to an hydrolyzed diet or a novel-source protein diet, treated for dysbiosis (with the use of antibiotics, probiotics or fecal transplant) as well as the use of one or combined immunosuppressants.

When available, *Leishmania* serology titers were detailed. Furthermore, if animals had already undergone a PCR test for *Leishmania* as part of their clinical investigation at the veterinary teaching hospital, the results were also documented.

During their follow-up at the Veterinary Teaching Hospital, dogs included were submitted to gastroduodenoscopy and/or colonoscopy.

Due to the intention of performing a retrospective study, the slides corresponding to biopsies of the selected dogs were retrieved from the sample bank, reexamined and submitted to an immunohistochemical technique for *Leishmania infantum*.

The immunohistochemistry technique was performed using the EnVision FLEX, High pH kit (Agilent Dako, REF K8000). For all samples, 3 micrometer tissue sections were used, mounted on adhesive slides. Sections underwent deparaffinization, hydration and epitope retrieval in EDTA buffer (pH 9) using Agilent Dako PT Link, Pre-Treatment Module for Tissue Specimens for 20 minutes at 96 degrees Celsius. Slides were then washed in distilled water twice for 5 minutes each and incubated in EnVision FLEX Peroxidase-Blocking Reagent (Agilent Dako kit) for 15 minutes, followed by washing in phosphate buffered saline (PBS) twice for 5 minutes each. Then, sections were incubated in the primary antibody (BioSystems, REF 44953), diluted at 1:25 in EnVision FLEX Antibody Diluent (Agilent Dako kit) for 60 minutes. After incubation and washing in PBS twice for 5 minutes each, the secondary antibody was applied (ready-to-use, BioSystems, REF 44960) for 30 minutes, followed by another washing step (as before). Sections were then incubated in EnVision/HRP polymer (Agilent Dako kit) for 30 minutes, followed by a subsequent PBS wash, as previously described. Subsequently, the sections were incubated in EnVision FLEX DAB + Chromogen (Agilent Dako kit), diluted in EnVision FLEX Substrate Buffer (Agilent Dako kit) at the dilution recommended by the supplier. After the addition of DAB, the slides were washed in distilled water, then stained with Harris' Hematoxylin for 1 minute and washed again in distilled water, before dehydration and mounting.

After these last steps, the slides were observed to verify whether they were positive or negative for amastigotes of *Leishmania*. As mentioned, positivity is reflected by a brownish color as shown in figures 1 and 2. As a positive control, a slide obtained from a lesion in the oral cavity of a cat naturally infected with *Leishmania infantum* was used. This slide underwent the same immunohistochemical technique.

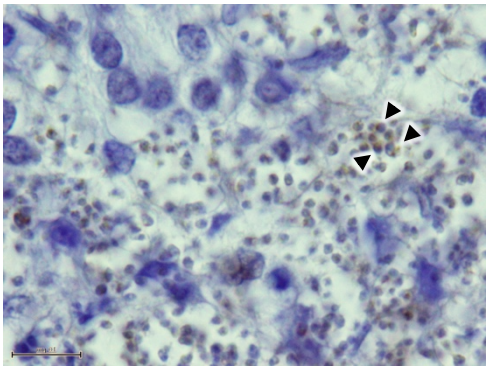


Figure 1 - Positive Control: Cat's Oral Lesion. Arrows on *Leishmania* amastigotes (100x objective; 10x eyepiece).

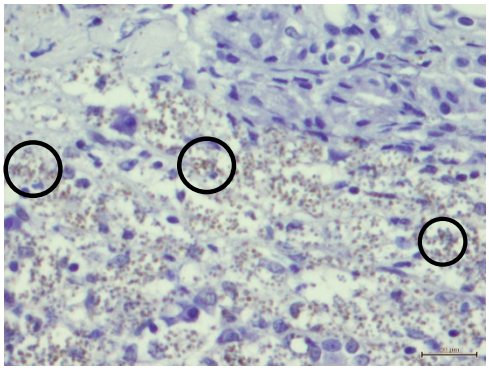


Figure 2 - Positive Control: Cat's Oral Lesion. *Leishmania* amastigotes circled (40x objective; 10x eyepiece).

In order to confirm and characterize the presence of chronic inflammation, an histopathological evaluation of the slides was performed. This evaluation included morphological and inflammatory criteria based on the *American College of Veterinary*

Internal Medicine (*ACVIM*) *Consensus Statement* (Washabau et al. 2010). The duodenum and ileum were evaluated according to the same criteria. The morphological criteria evaluated for these two intestinal segments were the ratio of villous length to crypt depth, villus stunting, epithelial injury, crypt distension, lacteal dilation, mucosal fibrosis and villus fusion. As for the inflammatory criteria, the parameters evaluated for these two segments were the presence of intraepithelial lymphocytes, the presence of lymphocytes, plasma cells, eosinophils and neutrophils in the lamina propria and the presence of *Leishmania* amastigotes in the sample. With regard to the colon, the morphological criteria evaluated were the presence of surface epithelial injury, crypt hyperplasia, crypt dilation and distortion, and mucosal fibrosis and atrophy. As for the inflammatory criteria, the parameters evaluated were the presence of lymphocytes, plasma cells, eosinophils, neutrophils and macrophages in the lamina propria and the presence of *Leishmania* amastigotes in the sample.

In order to proceed with the evaluation of the previously mentioned parameters, a classification was assigned to each one of the intestinal section alterations. A classification of the type ''X:Y'' was assigned to the morphological criteria ''ratio of villous length to crypt depth'' in the duodenum and ileum. The values ''X'' and ''Y'' corresponded to a number from 1 to 3 based on the verified proportion. The parameter ‘’villous stunting’’ can be inferred from the parameter ‘’ratio of villous length to crypt depth’’ since a ratio of 2:1 is considered normal and ratios below that are indicative of atrophy. For the morphological parameters ‘’villous stunting’’, ‘’epithelial injury’’ and ‘’villous fusion’’ from the duodenum and ileum criteria and for the ‘’surface epithelial injury’’ and ‘’crypt hyperplasia’’ from the colon criteria, a qualitative classification was assigned which could be ‘’yes’’, ‘’no’’ or ‘’not determined’’ according to the presence or absence of the alteration. For the remaining morphologic criteria and all inflammatory criteria, a value from zero to six according to the gravity of the lesions was assigned, with six corresponding to a worse alteration. This final classification value was obtained based on the scheme shown in figure 3. Each parameter was assigned with one or more plus signs (+) (maximum up to three) or a minus sign (-) if the alteration was not present or was impossible to assess. A minus sign corresponds to zero points, a plus sign corresponds to one point, two plus signs corresponds to two points and three plus signs corresponds to three points. However, as some parameters do not have a diffuse distribution throughout the sample, appearing in clusters, in addition to being important to classify the severity of the alteration with minus and plus signs, it was also important to characterize the frequency of appearance of such alterations. For that reason, the letters ‘’F’’ of frequent, ‘’O’’ of occasional and ‘’R’’ of rare were also assigned to each of the parameters, with ‘’F’’ corresponding to three points, ‘’O’’ corresponding to two points and ‘’R’’ corresponding to one point. Alterations with diffuse distribution, in particular, the presence of lymphocytes and plasma cells in the lamina propria, were assigned the letter ‘’F’’ of frequent, which

corresponds to three points. The final classification was obtained by adding both the severity classification and the frequency classification. After calculating the final classification of every criteria for each slide, the values from 0 to 6 of each of the variables were transformed into classes. Alterations with ratings of 1 and 2 were classified as ‘’mild’’, those with ratings of 3 and 4 were classified as ‘’moderate’’ and those with ratings of 5 and 6 were classified as ‘’severe’’. Criteria with a final score of 0 were classified as ‘’absent’’.

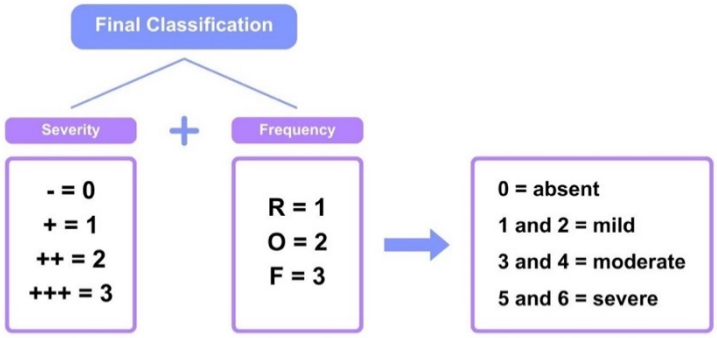


Figure 3 - Final Classification Scheme for Morphologic and Inflammatory Alterations.

The absolute and relative frequency of animals in which the alterations were verified as well as the proportion of animals with alterations classified as mild, moderate or severe were calculated using Microsoft Excel® for Mac, version 16.64.

For each animal, information regarding their age, gender, breed and reproductive status was collected. In addition to this data, through the medical records available in the veterinary hospital management software *GuruVet*, the animals’ history was compiled, including their presenting clinical signs, refractory clinical signs, the treatment to which each one was subjected and the results of analysis conducted up to one month before endoscopy, specifically the complete blood count, biochemistry analysis and the urinary protein/creatinine ratio. The survival time of each one of the dogs was also compiled.

3.3 – Results

The results are compiled as a table in Annex 3.

3.3.1 – Characterization of the population

A total of 11 animals were included in the study. Among them, 5/11 (45%) were females of which 4/5 (80%) were spayed. Of the 6/11 (55%) males, 2/6 (33%) were neutered, 3/6 (50%) were intact and 1/6 (17%) had unknown reproductive status.

Median age at diagnosis of chronic enteropathy was 5 years, with an interquartile range from 3 (Q1) to 8 (Q3).

Regarding breed, 2/11 (18%) were cross-breed, 2/11 (18%) were Yorkshire Terriers and there was one (1/11; 9%) animal from each of the following breeds: Portuguese Water Dog, Pomeranian, Golden Retriever, German Shepherd, Great Dane, Pinscher and Boxer.

3.3.2 – Clinical Signs

As part of inclusion criteria, all animals in this study had chronic gastrointestinal clinical signs. Of all the clinical signs presented, there were: diarrhea and/or unformed feces in all of the dogs (11/11; 100%), weight loss in 9/11 (82%) dogs and vomiting in 6/11 (55%) dogs. Three (3/11; 27%) dogs presented with hematochezia and anorexia, 2/11 (18%) had mucus in feces, abdominal spasm/pain and borborygmus and 1/11 (9%) presented hyporexia, abdominal distension, eructation and hematemesis. The absolute and relative frequencies of each clinical sign in these animals is shown in table 1.

Table 1 – Clinical Signs at Presentation

Clinical Sign	Absolute Frequency	Relative Frequency (%)
Diarrhea / Unformed feces	11	100
Weight Loss	9	82
Vomiting	6	55
Hematochezia	3	27
Anorexia	3	27
Mucus in the feces	2	18
Abdominal Spasm and Pain	2	18
Borborygmus	2	18
Hyporexia	1	9
Abdominal Distension	1	9
Eructation	1	9
Hematemesis	1	9

In addition to the gastrointestinal signs, 6/11 (55%) dogs also presented with non-gastrointestinal clinical signs and/or concurrent diseases. One of the animals had chronic bronchitis, another had occasional episodes of gagging, two dogs occasionally experienced regurgitation episodes, another one displayed some degree of ataxia and one had incontinence and previous diagnosis of atopic dermatitis.

After treatment, none of these animals had a full recovery, remaining with some refractory signs. Table 2 summarizes the absolute and relative frequencies of these clinical signs. Similarly to what was verified prior to treatment, diarrhea and vomiting were still the most frequent clinical signs with 10/11 (91%) and 6/11 (55%) dogs affected, respectively. However, the frequency of dogs with hematochezia doubled after treatment, with this clinical sign also affecting 6/11 (55%) dogs. Five (5/11; 45%) dogs still presented weight loss and

2/11 (18%) had mucus in the feces after treatment. Only one (1/11; 9%) dog was affected for each of the remaining clinical signs (anorexia, abdominal spasm/pain, borborygmus, eructation and nausea).

Table 2 – Refractory Clinical Signs

Clinical Sign	Absolute Frequency	Relative Frequency (%)
Diarrhea / Unformed feces	10	91
Vomiting	6	55
Hematochezia	6	55
Weight Loss	5	45
Mucus in the feces	2	18
Anorexia	1	9
Abdominal Spasm and Pain	1	9
Borborygmus	1	9
Eructation	1	9
Nausea	1	9

3.3.3 – Laboratory Findings

Seven (7/11; 64%) dogs underwent a complete blood count conducted up to one month before endoscopy. Among them, 3/7 (43%) dogs presented anemia and also 3/7 (43%) had leukocytosis due to neutrophilia, with one of them presenting monocytosis along with neutrophilia. Regarding platelet count, 1/7 (14%) dog exhibited thrombocytosis while another one (1/7; 14%) presented thrombocytopenia.

Eight (8/11; 73%) dogs underwent biochemical analyses. Albumin and total proteins were assessed in 7/11 (64%) dogs, revealing hypoalbuminemia in 4/7 (57%) dogs and hypoproteinemia in 3/7 (43%). Gamma-glutamyl transferase was evaluated in 2/11 (18%) dogs, with 1/2 (50%) showing an increase of this parameter. The concentration of urea was evaluated in 3/11 (27%) dogs, with 1/3 (33%) presenting levels slightly above the maximum limit. Cobalamin levels were examined in 6/11 (55%) dogs. In 2/6 (33%), levels surpassed the maximum limit, while in another 2/6 (33%), levels were below the minimum threshold. Finally, the urinary protein/creatinine ratio was assessed in 2/11 (18%) dogs and only 1/2 (50%) exhibited elevated levels.

3.3.4 – Serology and Polymerase Chain Reaction for *Leishmania*

An ELISA test was performed on 5/11 (45%) dogs to detect and measure the presence of antibodies anti-*Leishmania* in dog serum. One (1/5; 20%) of the dogs tested positive for *Leishmania* with a ratio of 4,24 equivalent to 1/320 on indirect

immunofluorescence. Duodenum samples from the positive dog were submitted to real-time PCR for *Leishmania infantum* after performing endoscopy with biopsy and the results came back negative.

3.3.5 – Histopathological Evaluation

All dogs underwent endoscopic examination. Specifically, 5/11 (45%) dogs underwent gastroduodenoscopy, 3/11 (27%) underwent colonoscopy, and the remaining 3/11 (27%) underwent both procedures. In the course of these endoscopic examinations, a total of 8 slides containing duodenal samples, 4 slides with ileal samples, and 6 slides with colonic samples were obtained.

3.3.5.1 – Duodenum

3.3.5.1.1 - Morphologic Criteria

Table 3 demonstrates the ratio of villous length to crypt depth in each of the duodenum samples. Villous stunting was present in 5/8 (63%) samples. In one of the remaining samples there was no villous stunting and in the other two it was not possible to verify the presence or absence of atrophy. Epithelial injury was only visible in 1/8 (13%) sample and villous fusion was present in 4/8 (50%) samples.

Table 3 – Ratio of Villous Length to Crypt Depth and Villous Stunting in the Duodenum

Sample	Ratio of Villous Length to Crypt Depth	Villous Stunting
1	2:1 – 1:1	No
2	1:1 – 1:2	Yes
3	1:2 – 1:3	Yes
4	Not Determined	Not Determined
5	1:1 – 1:2	Yes
6	1:1	Yes
7	1:1 – 1:2	Yes
8	Not Determined	Not Determined

Concerning the remaining morphological variables (table 4), crypt distension, lacteal dilation and mucosal fibrosis were present in 3/8 (38%), 7/8 (88%) and 2/8 (25%) samples, respectively. About crypt distension, the number of animals with mild, moderate and severe lesions was 1/8 (13%) for all three categories. In 5/8 (63%) animals the alteration was classified as ‘’absent’’. As for lacteal dilation, 1/8 (13%) dog was classified with a mild lesion,

4/8 (50%) had a moderate lesion, 2/8 (25%) had a severe lesion and in 1/8 (13%) that alteration was absent. Finally, concerning mucosal fibrosis, this alteration was absent in 6/8 (75%) dogs, while the remaining 2/8 (25%) had fibrosis lesions classified as moderate.

Table 4 – Crypt Distension, Lacteal Dilation and Mucosal Fibrosis in the Duodenum

Criteria	AF	RF (%)	Mild (%)	Moderate (%)	Severe (%)	Absent (%)
Crypt Distension	3	38	13	13	13	63
Lacteal Dilation	7	88	13	50	25	13
Mucosal Fibrosis	2	25	0	25	0	75

AF – Absolute Frequency; RF – Relative Frequency.

3.3.5.1.2 - Inflammatory Criteria

Intraepithelial lymphocytes were observed in 6/8 (75%) duodenum samples. Both lymphocytes and plasma cells were identified in the lamina propria of all (8/8; 100%) of these samples. Eosinophils were found in the lamina propria of 5/8 (63%) samples while, with the lowest frequency, only 3/8 (38%) samples presented neutrophils in the lamina propria.

Regarding intraepithelial lymphocytes, the infiltrate was considered mild in 1/8 (13%) animal, moderate in 4/8 (50%), severe in 1/8 (13%) and was absent in 2/8 (25%). As for the presence of lymphocytes in the lamina propria, in 6/8 (75%) samples the infiltrate was classified as moderate and in 2/8 (25%) it was classified as severe. Concerning plasma cells in the lamina propria, in 3/8 (38%) samples the infiltrate was classified as moderate while in 5/8 (63%) it was classified as severe. Regarding eosinophils in the lamina propria, the infiltrate was classified as mild in 3/8 (38%) samples, the number of animals with moderate and severe infiltrate was 1/8 (13%) for both classes and the infiltrate was absent in 3/8 (38%) samples. Finally, concerning neutrophils in the lamina propria, this infiltrate was mild in 1/8 (13%) sample, moderate in 2/8 (25%) and absent in 5/8 (63%). This information is summarized in table 5.

Table 5 – Inflammatory Criteria in the Duodenum

Criteria	AF	RF (%)	Mild (%)	Moderate (%)	Severe (%)	Absent (%)
Intraepithelial Lymphocytes	6	75	13	50	13	25
Lymphocytes in the LP	8	100	0	75	25	0
Plasma cells in the LP	8	100	0	38	63	0
Eosinophils in the LP	5	63	38	13	13	38
Neutrophils in the LP	3	38	13	25	0	63

LP - Lamina Propria; AF – Absolute Frequency; RF – Relative Frequency.

3.3.5.2 – Ileum

3.3.5.2.1 – Morphologic Criteria

In this intestinal segment, villous stunting was present in all (4/4; 100%) of the samples (table 6). Both epithelial injury and villous fusion were present in 3/4 (75%) samples.

Table 6 – Ratio of Villous Length to Crypt Depth and Villous Stunting in the Ileum

Sample	Ratio of Villous Length to Crypt Depth	Villous Stunting
1	1:2	Yes
2	1:2 – 1:3	Yes
3	1:2	Yes
4	1:1 – 1:2	Yes

Concerning the remaining morphological variables (table 7), both crypt distension and mucosal fibrosis were present in 3/4 (75%) samples and lacteal dilation was identified in all of the samples (4/4; 100%).

The lesion crypt distension was classified as moderate in 3/4 (75%) of the ileum samples and was absent in the remaining one (1/4; 25%). As for lacteal dilatation, the lesion was also considered moderate in 3/4 (75%) samples and, in the remaining sample (1/4; 25%), the lesion was considered severe. With regard to mucosal fibrosis, 2/4 (50%) animals had lesions classified has moderate, 1/4 (25%) had lesions classified has severe and this lesion was absent in the remaining sample (1/4; 25%).

Table 7 – Crypt Distension, Lacteal Dilation and Mucosal Fibrosis in the Ileum

Criteria	AF	RF (%)	Mild (%)	Moderate (%)	Severe (%)	Absent (%)
Crypt Distension	3	75	0	75	0	25
Lacteal Dilation	4	100	0	75	25	0
Mucosal Fibrosis	3	75	0	50	25	25

AF – Absolute Frequency; RF – Relative Frequency.

3.3.5.2.2 – Inflammatory Criteria

In the ileum, intraepithelial lymphocytes were only identified in 1/4 (25%) sample. Lymphocytes, plasma cells and neutrophils were identified in the lamina propria of all samples (4/4; 100%) and eosinophils were observed in 3/4 (75%) samples.

The infiltration of the epithelium with lymphocytes was classified as mild in the only (1/4; 25%) sample where those cells were identified as it was absent in the remaining 3/4

(75%). The number of animals with moderate and severe infiltration of lymphocytes in the lamina propria was 2/4 (50%) for both classes. As for plasma cells in the lamina propria, the infiltrate was classified as moderate in 3/4 (75%) samples and as severe in the remaining one (1/4; 25%). A mild eosinophilic infiltrate was present in the lamina propria of 1/4 (25%) sample, a moderate infiltrate was present in 2/4 (50%) samples, and there was no infiltrate in one (1/4; 25%) of the samples. The infiltration of neutrophils in the lamina propria was classified as mild in 1/4 (25%) sample and moderate in the remaining 3/4 (75%) samples. This information is summarized in table 8.

Table 8 – Inflammatory Criteria in the Ileum

Criteria	AF	RF (%)	Mild (%)	Moderate (%)	Severe (%)	Absent (%)
Intraepithelial Lymphocytes	1	25	25	0	0	75
Lymphocytes in the LP	4	100	0	50	50	0
Plasma cells in the LP	4	100	0	75	25	0
Eosinophils in the LP	3	75	25	50	0	25
Neutrophils in the LP	4	100	25	75	0	0

LP - Lamina Propria; AF – Absolute Frequency; RF – Relative Frequency.

3.3.5.3 – Colon

3.3.5.3.1 – Morphologic Criteria

Regarding the morphologic criteria, epithelial injury was found in 2/6 (33%) of the colon samples and crypt hyperplasia was present in 4/6 (67%) samples. The lesion crypt dilation and distortion was identified in all of the animals (6/6; 100%) and, in 5/6 (83%) mucosal fibrosis and atrophy was also present (table 9).

Crypt dilation and distortion was classified as moderate in 5/6 (83%) samples and as severe in the remaining one (1/6; 17%). As for mucosal fibrosis and atrophy, this lesion was classified as moderate in 3/6 (50%) samples, as severe in 2/6 (33%) and it was absent in the remaining sample (1/6; 17%).

Table 9 – Crypt Dilation and Distortion and Mucosal Fibrosis and Atrophy in the Colon

Criteria	AF	RF (%)	Mild (%)	Moderate (%)	Severe (%)	Absent (%)
Crypt Dilation and Distortion	6	100	0	83	17	0

Mucosal Fibrosis and Atrophy	5	83	0	50	33	17

AF – Absolute Frequency; RF – Relative Frequency.

3.3.5.3.2 – Inflammatory Criteria

In all colon samples (6/6; 100%), lymphocytes, plasma cells and macrophages were identified in the lamina propria. Eosinophils were present in the lamina propria of 2/6 (33%) samples and neutrophils were present in the lamina propria of 3/6 (50%) samples, as demonstrated in table 10.

The lymphocyte infiltrate in the lamina propria was classified as moderate in 4/6 (67%) of the colon samples and as severe in the remaining 2/6 (33%). The opposite was verified with the plasmacytic infiltrate in the lamina propria, since it was classified as moderate in 2/6 (33%) samples and as severe in 4/6 (67%). A moderate eosinophilic infiltrate in the lamina propria was present in 2/6 (33%) samples and, in the remaining 4/6 (67%), these cells were not detected. In regard to neutrophils in the lamina propria, the infiltrate was classified as mild in 1/6 (17%) sample, moderate in 2/6 (33%) and was absent in 3/6 (50%) colon samples. Concerning the presence of macrophages in the lamina propria, the infiltrate was classified as mild in 1/6 (17%) sample and as moderate in the remaining 5/6 (83%).

Table 10 – Inflammatory Criteria in the Colon

Criteria	AF	RF (%)	Mild (%)	Moderate (%)	Severe (%)	Absent (%)
Lymphocytes in the LP	6	100	0	67	33	0
Plasma cells in the LP	6	100	0	33	67	0
Eosinophils in the LP	2	33	0	33	0	67
Neutrophils in the LP	3	50	17	33	0	50
Macrophages in the LP	6	100	17	83	0	0

LP - Lamina Propria; AF – Absolute Frequency; RF – Relative Frequency.

3.3.6 – Immunohistochemistry for *Leishmania*

Following the immunohistochemistry technique, none of the slides exhibited the brownish color observed in the control slides (Figures 1 and 2) when viewed under an optical microscope. This indicates that all samples tested across different intestinal segments were

negative for *Leishmania infantum*. Figures 4 and 5 correspond to one of the studied slides, specifically a small intestine slide, demonstrating a negative result.

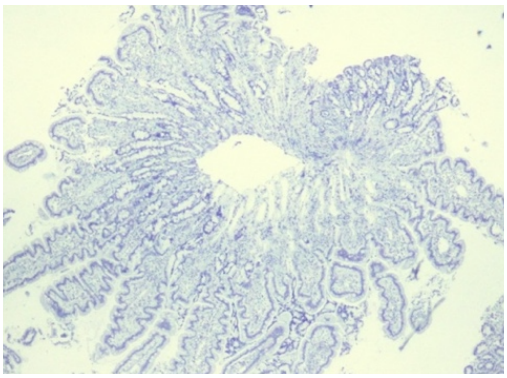


Figure 4 - Example of Negative Result in Immunohistochemistry for *Leishmania* (2x objective; 10x eyepiece).

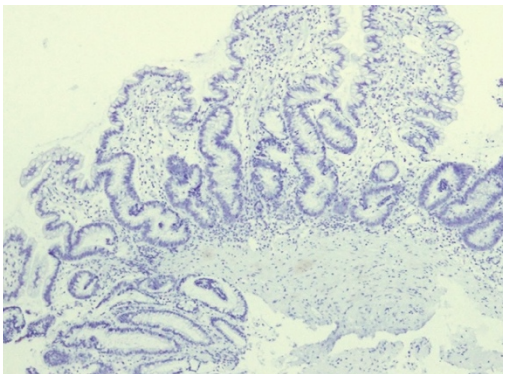
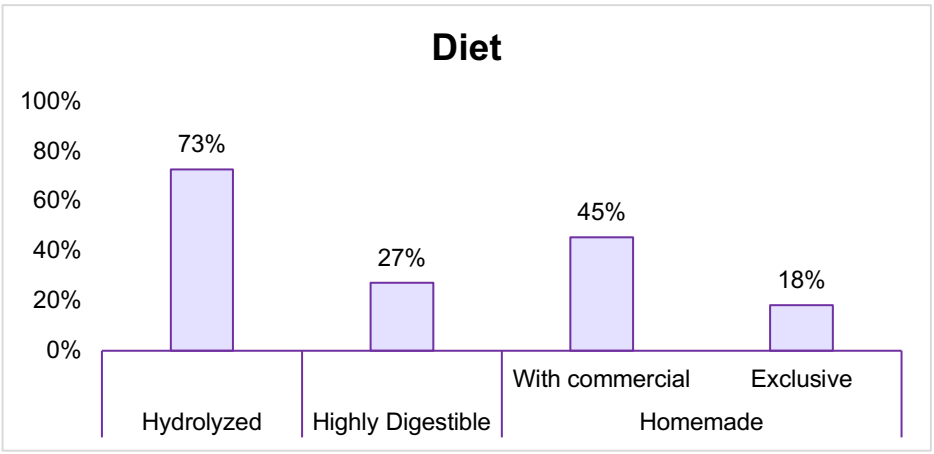


Figure 5 - Example of Negative Result in Immunohistochemistry for *Leishmania* (4x objective; 10x eyepiece).

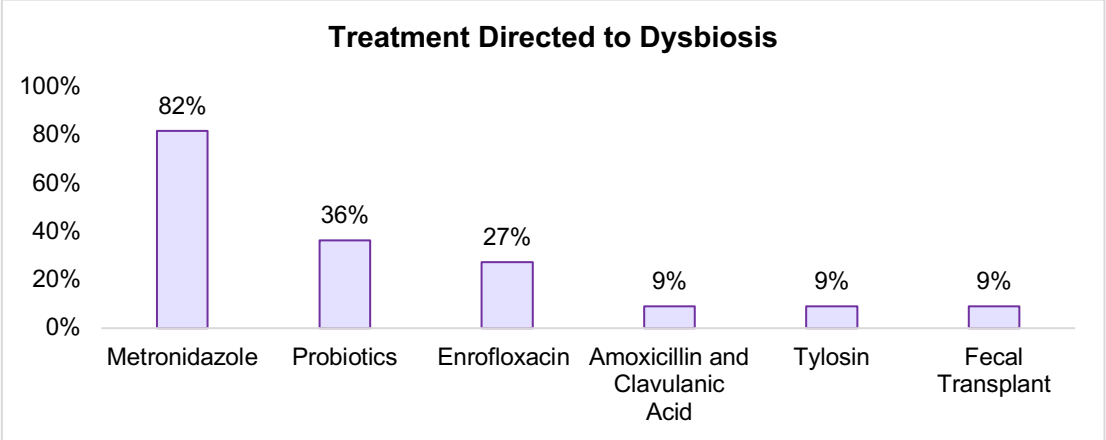
3.3.7 – Treatment

All animals initially underwent a dietary modification prescribed either by the Veterinary Teaching Hospital staff or by the hospital/clinic referring the case. Not all of them always maintained the same initial diet. One dog initially started treatment with a homemade diet, subsequently transitioning to a highly digestible commercial one. Another dog started a hydrolyzed commercial diet and later adopted a combination of hydrolyzed and highly digestible diets, later on, this same dog transitioned to a regimen incorporating both a highly digestible commercial diet and homemade components. Graph 1 specifies the diet options followed by these animals. Eight (8/11; 73%) dogs at some point followed a hydrolyzed diet, which was the option most chosen by the clinicians. Three (3/11; 27%) dogs were fed a highly digestible diet and 7/11 (64%) were fed a homemade diet (duck and sweet potato), as either an exclusive diet (2/11; 18%) or alternating with a commercial diet (5/11; 45%). Concerning regimens combining both homemade and commercial diets, 3/11 (27%) dogs were administered a hydrolyzed commercial diet with homemade food, while 2/11 (18%) dogs received a mixture of highly digestible commercial diet with homemade components.



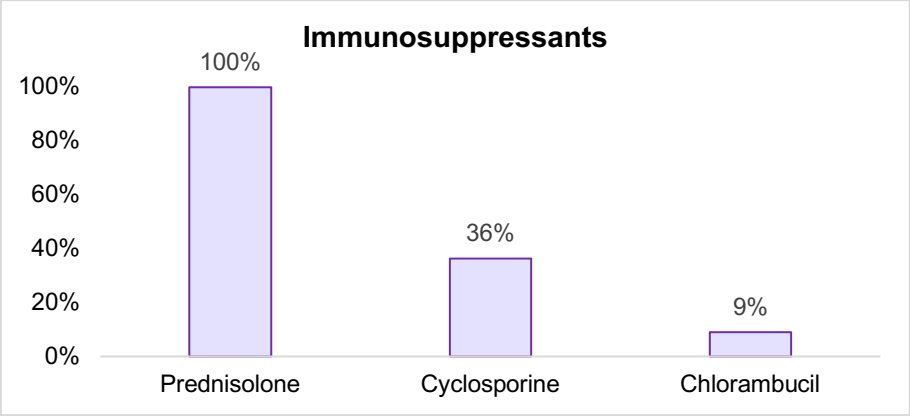
Graph 1 – Diet Trial

Regarding treatment directed to modulate microbiota, all dogs (11/11; 100%) received antibiotic treatment. The most frequently prescribed medication was metronidazole which was given to 9/11 (82%) dogs, followed by probiotics which were administered to 4/11 (36%) dogs. Apart from metronidazole, other antibiotics that were administered to the animals in this study were enrofloxacin (3/11 dogs; 27%), tylosin (1/11 dogs; 9%) and amoxicillin with clavulanic acid (1/11 dogs; 9%). Fecal transplantation was a strategy only applied to one (1/11; 9%) of the eleven dogs. Graph 2 summarizes the aforementioned information.



Graph 2 - Treatment Directed to Dysbiosis

About immunosuppressants, all animals in this study were treated with at least one immunosuppressant. Prednisolone was administered to all dogs (11/11; 100%) in this study. However, 4/11 (36%) dogs required the addition of a second immunosuppressant to lessen the negative effects brought on by long-term corticosteroid use. As evidenced in graph 3, cyclosporine was the second immunosuppressant of choice to associate with prednisolone, being administered to 4/11 (36%) dogs, while chlorambucil was only given to 1/11 dog (9%) and, later, due to the onset of anorexia and pancreatitis following the fourth dose of this medication, it was replaced with cyclosporine.



Graph 3 - Treatment with Immunosuppressants

Apart from these 3 treatment phases, many dogs required another type of medication to manage gastrointestinal signs. Four dogs (4/11; 36%) required treatment for pain management (methadone in 2/11, 18%; fentanyl in 1/11, 9%; paracetamol in 1/11, 9%; and buprenorphine in 1/11, 9%). Seven (7/11; 64%) dogs required treatment with an anti-emetic, being the ones administered: ondansetron (3/11; 27%), maropitant (7/11; 64%) and methaclopramide (3/11; 27%). Omeprazole was administered to 6/11 (55%) animals and sucralfate to 3/11 (27%). One (1/11; 9%) dog required cyanocobalamin supplementation and another one (1/11; 9%) required spironolactone due to the presence of ascites secondary to protein-losing enteropathy.

3.3.8 – Survival Time

Three (3/11; 27%) of the eleven animals died, 2/3 (67%) were euthanized, and 1/3 (33%) died following a severe episode of diarrhea, hematochezia, and vomiting. The exact cause of death remains unknown, as a necropsy was not performed, highlighting a potential undiagnosed underlying condition. Of the other 8/11 (73%) dogs, 5/8 (63%) were lost to follow-up and the remaining 3/8 (38%) were still alive, 1 year (diagnosis in 2022), 3 years (diagnosis in 2020) and 5 years (diagnosis in 2018) after diagnosis.

3.4 – Discussion

This study tried to clarify the role of leishmaniosis as a differential diagnosis of refractory canine chronic enteropathy. For this purpose, cases of RCE were detailed. The sample was constituted by a small number of cases of chronic enteropathy followed in the internal medicine service at the Veterinary Teaching Hospital. The median age at diagnosis was 5 years. The onset of refractory IBD is typically in middle age (Hall and Day 2017) but it

is worth noting that the definition of middle age can vary depending on the size of the dog breed. The observed median in our study, which included a diverse range of breeds, might be slightly below the expected range. This variation could be influenced by the diversity of breed sizes within our sample. Additionally, the small size of our sample should be taken into consideration when interpreting these findings. This may lead to a greater influence of atypical cases on the median than would be expected in a larger sample.

Notably, every dog in the study presented with diarrhea and/or soft stools. Among the additional clinical signs observed, weight loss and vomiting emerged as the two most common ones, which is consistent with what is reported in the literature (Jergens and Simpson 2012; Hall and Day 2017). Regarding the laboratory abnormalities identified in the studied animals, most of them could be consistent with both the diagnosis of chronic enteropathy and leishmaniosis, although, other differentials may be considered. For example, three dogs displayed anemia and neutrophilia, alterations commonly described in the literature for both chronic enteropathy and leishmaniosis (Ciaramella et al. 1997; Koutinas et al. 1999; Freitas et al. 2012; Jergens and Simpson 2012; Hall and Day 2017; Baneth, Petersen, et al. 2022). However, beyond these two diseases, there are additional differentials that should be taken into consideration. Non-protozoal infections, neoplasia, tissue damage or necrosis may all be accompanied with neutrophilia, for example (DeClue and Spann 2017; Thompson 2018).

According to its classification, anemia can have a variety of causes. To name a few, regenerative anemia is often associated with acute or chronic bleeding, such as gastrointestinal bleeding, coagulation disorders, trauma, hematuria and other parasites. On the other hand, non-regenerative anemia, typically seen in leishmaniosis, can be associated to chronic diseases, medication side effects, endocrine disease, iron deficiency and other infectious causes (Thompson 2018). Hypoalbuminemia was identified in four animals and it is also a laboratory abnormality that can arise in both of these diseases (Ciaramella et al. 1997; Koutinas et al. 1999; Allenspach et al. 2007; Freitas et al. 2012; Hall and Day 2017; Baneth, Petersen, et al. 2022). This protein may, however, be low because of decreased production brought on by liver disease, which was not verified in any of the studied animals, due to any inflammation in the body as it corresponds to a negative acute phase protein or due to renal loss, which would be a possible scenario as one of the studied dogs presented an increased urinary protein/creatinine ratio (Burton 2017). Thrombocytopenia is not so commonly observed in chronic enteropathy (Hall and Day 2017) and it was only present in one of the studied animals, which coincided with the dog positive for leishmaniosis. This dog was also one of the animals with neutrophilia and hypoalbuminemia, supporting that these laboratory findings may have been a consequence of this parasitic infection.

In order to be included in this study all dogs were required to have undergone a sequential treatment, starting with dietary modification, followed by targeted therapy for dysbiosis, and finally, treatment with immunomodulators. Regarding diet, the clinicians' preferred choice was hydrolyzed diet, aligning with recent studies that highlighted the effectiveness of such diets in reducing inflammatory response and achieving clinical improvement (Marchesi et al. 2017; Kathrani and Hall 2019). In fact, hydrolyzed diets have demonstrated greater efficacy in long-term management, being associated with fewer relapses when compared to highly digestible diets (Mandigers et al. 2010), which were only administered to 3 animals in this study. All animals in this study underwent antibiotic therapy, with metronidazole being the clinicians' preferred choice, likely attributed to its immunomodulatory effects on cellular immunity (Hall and Day 2017). Regarding probiotics and fecal transplantation, the administration percentage of these alternatives in this study was slightly lower than desired, given that the long-term goal is to utilize these therapies as alternatives to antibiotics in modulating bacterial populations (Cerquetella et al. 2020). All animals underwent prednisolone therapy, as glucocorticoids are the most common choice as immunomodulators in dogs (Hall and Day 2017). Four of them required the addition of a second immunosuppressor, with clinicians preferring ciclosporin, despite it being an expensive option (Hall and Day 2017). This cytotoxic drug has demonstrated promising effects, such as its specific activity on T lymphocytes (Allenspach et al. 2006).

All cases had a confirmed diagnosis of chronic enteropathy through histopathological analysis of samples. Both morphological and inflammatory criteria consistently indicated the presence of chronic inflammation in every case. All slides with samples were reviewed by the same pathologist based on the *ACVIM Consensus Statement* (Washabau et al. 2010) to reduce inter-observer variability and subjectivity in their interpretation.

Even though the main clinical sign of these animals was diarrhea, an attempt was still made to establish a relation between refractory chronic enteropathy and leishmaniosis as some case reports described similar situations where the sole manifestations of leishmaniosis were gastrointestinal (Ferrer et al. 1991; Ruiz et al. 2015; Vila et al. 2016; Ayala et al. 2017; López et al. 2022). The results of the immunohistochemical technique for *Leishmania* in this study were all negative. One of the animals was positive for *Leishmania* in a serological test, however, after performing endoscopy with biopsy, the duodenum samples were submitted to real-time PCR for *Leishmania infantum* and the results came back negative, therefore, it was anticipated that the sample would also test negative on immunohistochemistry. Although the results obtained in this project are not compatible with those mentioned above, those case reports exacerbate the need to consider leishmaniosis as a differential diagnosis for refractory chronic diarrhea, especially in endemic areas.

Immunohistochemistry was the technique employed for this project since it has been previously shown to be effective for diagnosis (Sílvio Coura Xavier et al. 2006; Amato et al. 2009; Queiroz et al. 2010; Shirian et al. 2014). This technique provides optimal visualization of the parasite due to the high degree of contrast between the parasites and the host tissue (Queiroz et al. 2010). In comparison to the traditional histological method, the identification of *Leishmania* through immunohistochemistry is more easily interpretable, making it accessible to less experienced observers at lower magnifications (Kenner et al. 1999). This approach has demonstrated higher sensitivity than histopathology in several studies (Shirian et al. 2014; Ferreira et al. 2022). Immunohistochemistry is commonly applied to fixed tissues (Kenner et al. 1999), enabling the visualization of intact amastigotes within the tissue and facilitating the correlation of parasites with associated lesions, which is not possible with some molecular methods like PCR (Ramos-Vara et al. 2009; Webster et al. 2010; Menezes et al. 2013). Furthermore, this technique does not require the use of expensive equipment and the resulting stained slides can be stored for several months (Ramos-Vara et al. 2009).

However, immunohistochemistry involves numerous repetitive, operator-dependent steps, increasing the likelihood of technical errors and, thus, inappropriate staining (Taylor and Shi 2014). Inappropriate staining in immunohistochemistry can be categorized into distinct types. Firstly, the absence of staining may occur in both the test tissue and the positive control, often attributed to issues within the staining procedure itself or to the antigen retrieval process (Ramos-Vara et al. 2008; Taylor and Shi 2014). Alternatively, absence or weak staining in the test tissue with appropriate positive staining in the control, is typically linked to pre-analytical steps. This can result from improper tissue fixation, processing or pretreatment, including antigen retrieval, or a combination of these (Taylor and Shi 2014). Background staining can also be present and consists of positive staining unrelated to an antibody-antigen reaction (Taylor and Shi 2014). Factors contributing to background staining include the presence of endogenous peroxidase, poorly fixed tissue and necrotic areas, contamination of the antibody solution, incomplete removal of paraffin or an excess concentration of both the primary antibody or the chromogen (Ramos-Vara et al. 2009; Taylor and Shi 2014). Additionally, artifactual staining may be observed, characterized by the presence of undissolved precipitates of chromogen or counterstain, endogenous pigments, or microbial contaminants (Taylor and Shi 2014).

In the specific context of this study, appropriate staining of the positive control was observed, while all samples exhibited an absence of staining. Therefore, in the event that these are not indicative of negative results but rather limitations of the technique, it would be expected that these limitations are associated with pre-analytic steps. One of the pre-analytical variables under consideration is fixation. Formalin provides excellent preservation of tissue architecture by inhibiting cellular processes, preventing tissue degradation and

effectively eliminating pathogens within lesions (Webster et al. 2009). Despite being crucial for later antigen detection in immunohistochemistry, it is essential to acknowledge that formalin-induced protein cross-links are a consequence of fixation and may potentially alter or hide epitopes, thereby preventing immunohistochemical reactions (Webster et al. 2010). Antigen retrieval techniques are employed to reverse these changes produced during fixation (Ramos-Vara et al. 2009). Several studies have demonstrated that prolonged formalin fixation is not a significant limiting factor for the majority of antibodies used in immunohistochemistry diagnostics, as it does not significantly inhibit immunoreactivity in paraffin sections (Ramos-Vara et al. 2000; Ramos-Vara et al. 2001; Ramos-Vara et al. 2002; Ramos-Vara et al. 2003; Webster et al. 2009; Webster et al. 2010). However, overfixation can result in a lack of staining. Therefore, it is generally recommended that formalin fixation should not exceed 48 hours (Taylor and Shi 2014). Contrary to common belief, Ramos-Vara et al. (2009) argues that underfixation is equally as detrimental or even worse than overfixation, challenging the idea that overfixation is the major concern. This poses a significant challenge, especially given the reduced turnaround times in diagnostic laboratories. Delayed fixation may lead to lack of staining due to autolysis (Taylor and Shi 2014). For that reason, immediate fixation, within 30 minutes of specimen removal, is recommended (Taylor and Shi 2014). In cases of overly large tissues, the fixative may only penetrate the periphery, leaving the central areas unfixed and raw. This can lead to coagulative fixation during tissue dehydration, causing variable staining (Webster et al. 2009; Taylor and Shi 2014). Occasionally, issues in tissue processing may also arise, including inadequate dehydration from aged alcohol reagents or the loss of heat-sensitive epitopes due to embedding in excessively hot paraffin or prolonged heating (Taylor and Shi 2014). Monitoring paraffin temperature is essential, not exceeding 56°C, to mitigate these concerns (Taylor and Shi 2014). Another limitation regarding immunohistochemistry involves the antigen retrieval step, specifically the lack of standardized methods and quality control across laboratories (Ramos-Vara et al. 2009; Kabiraj et al. 2015). The subjectivity in interpreting immunohistochemistry results, given the wide range of interobserver interpretations, is also noted as a limitation of the technique (Ramos-Vara et al. 2009; Ferreira et al. 2022).

In the specific context of canine leishmaniosis, the immunohistochemistry technique is frequently employed by various authors for disease diagnosis (Kenner et al. 1999; Solano-Gallego et al. 2004; S. C. Xavier et al. 2006; Lunedo et al. 2012). Even so, the sensitivity to detect *Leishmania* amastigotes seems to vary according to the clinical signs presented (Moreira et al. 2007). This was also evidenced in a study carried out by Queiroz et al. (2010) where immunohistochemistry was only positive for 28,6% (4/14) of dogs with few clinical signs, whereas it was positive for 91,7% (11/12) of dogs with numerous clinical signs. Similar

findings were obtained in a study by Solano-Gallego et al. (2004), where asymptomatic animals with positive PCR for *Leishmania* in the skin exhibited negative results in immunohistochemistry on skin biopsies. In contrast, symptomatic animals, also PCR-positive, showed positive results in immunohistochemistry. The authors suggested that this discrepancy could be attributed to a very low parasitic burden in the skin. These findings align with other studies (S. C. Xavier et al. 2006; Shirian et al. 2014) that have demonstrated the superior sensitivity of PCR, particularly in the detection of *Leishmania* in formalin-fixed paraffin-embedded tissues, when compared to immunohistochemistry.

However, similar to what is observed in immunohistochemistry, the PCR technique also experiences consequences and limitations associated with the fixation process. The formaldehyde fixation of tissue samples induces extensive crosslinking of all tissue components, resulting in highly fragmented nucleic acids (Satiroglu-Tufan et al. 2004; Castiglione et al. 2007; Huijsmans et al. 2010). These nucleic acids extracted from formalin-fixed paraffin embedded (FFPE) tissues are predominantly composed of small fragments, typically less than 300 bp (Dietrich et al. 2013). This degradation might be a challenge for molecular biological methods, such as PCR, which often require longer fragments for many analyses (Dietrich et al. 2013). Similar to observations in immunohistochemistry, studies devalue the impact of fixation on DNA quality, affirming that it resembles that of fresh tissue (Ludyga et al. 2012). Furthermore, addressing the challenge of DNA fragmentation, Castiglione et al. (2007) claims that quantitative PCR emerges as an advantage, enabling the use of relatively small quantities of genetic material, like the fragmented material obtained from FFPE tissues. Other limitations highlighted in PCR for FFPE tissues include the requirement for substantial tissue quantities to obtain the necessary DNA for analysis, leading to a significant sample loss (Jackson et al. 1990). Additionally, substances from the fixation or processing procedure may co-extract with the DNA, potentially causing amplification inhibition (Dietrich et al. 2013; Babouee Flury et al. 2014). When compared to immunohistochemistry, PCR does not allow for the correlation between the presence and localization of parasites with the observed lesions and tissue morphology (Ferreira et al. 2022), besides, PCR is more expensive due to the needed equipment, reagents, infrastructure, and the complexity of sample processing (Ferreira et al. 2022).

In general, the lower sensitivity of immunohistochemistry when compared to PCR is partially explained by the requirement that the antigen be in perfect structural condition in order for the antibodies to bind to it and yield a positive result (Amato et al. 2009). Whereas in PCR, where an amplification process is employed, the presence of a small gene sequence is enough to get a positive result (Amato et al. 2009). Bearing this in mind, in a case where the initial insult that gave rise to the inflammatory process had been *Leishmania* but where the antigens are no longer intact, immunohistochemistry would not detect any amastigotes.

However, in the presence of any tiny fragment of *Leishmania* genetic material, the PCR could still detect its presence and the result would come back positive. This could have occurred with the samples from the project's refractory cases, thus, it would have been ideal to have the option of applying molecular techniques on all samples and comparing the results. Therefore, some studies (Amato et al. 2009; Queiroz et al. 2010; Ferreira et al. 2022) suggest the association of techniques for diagnosis of leishmaniosis, using PCR in cases where the suspicion of leishmaniosis remains after a negative result in immunohistochemistry.

A technique that, to some extent, combines the benefits of both immunohistochemistry and PCR and could also have been applied is in situ hybridization (ISH). This technique applied to FFPE tissues has shown to be promising (Dinhopl et al. 2011; Frickmann et al. 2012; Menezes et al. 2013). It detects specific nucleic acid segments of *Leishmania*, while offering an advantage over PCR by allowing simultaneous correlation between parasite's presence and the observed lesion (Furtado et al. 2015). A notable advantage of ISH over immunohistochemistry is the higher specificity of ISH (Menezes et al. 2013). Nevertheless, ISH has lower sensitivity compared to PCR. This diminished sensitivity is due to the absence of DNA amplification, a feature inherent to PCR. In contrast, ISH relies on the direct detection of nucleic acids within the sample (Dinhopl et al. 2011; Ferreira et al. 2022).

As mentioned, the histological evaluation performed revealed the presence of chronic inflammation in all slides, confirming the diagnosis of chronic enteropathy. The evaluation of the inflammatory criteria of the slides evidenced that lymphocytes and plasma cells were the most abundant inflammatory cells in the lamina propria, which is in agreement to previous literature. According to Jergens and Simpson (2012), lymphoplasmacytic enteritis is the most frequently reported form of enteritis. However, in a first evaluation of the results obtained in histopathology, there seemed to be a tendency for an increase in plasma cells compared to lymphocytes, both in frequency and in severity.

In human medicine, the polarization of T helper cells in inflammatory diseases is well documented (Neurath et al. 1995; Boirivant et al. 1998; Heller et al. 2002; Neurath et al. 2002). This polarization is the key role for maintaining or disrupting the balance between tolerance and responsiveness (Neurath et al. 2002). Studies in murine models (Neurath et al. 1995; Boirivant et al. 1998; Heller et al. 2002) have demonstrated an association with Crohn's disease and a Th1-type immune response characterized by an increase in IL-12, IFN-γ and TNF-α, where anti-IL-12 antibody therapy or other Th1-inhibiting factors can prevent inflammation or reverse current inflammation. As for ulcerative colitis, there is an association with a Th2-type response with the production of IL-4 and IL-13 (Boirivant et al. 1998; Heller et al. 2002). According to a study carried out by Heller et al. (2005), T cells from

patients with ulcerative colitis produced more IL-13 than those from Crohn's disease patients or healthy individuals. In more recent studies, the possible involvement of another cell population in Crohn's disease, Th17 T cells, was demonstrated. This T cell is induced by IL-23 and is characterized by the secretion of IL-17, IL-6 and TNF-α (Hunter 2005).

The cytokine pattern in the inflamed intestinal mucosa has also been studied in veterinary medicine in an attempt to demonstrate a distinct T-helper lymphocyte profile. Jergens et al. (2009) reviewed a number of veterinary research which examined the role of different cytokines in the immunopathogenesis of canine IBD by a semiquantitative reverse transcriptase PCR technique. These studies presented a mixed cytokine pattern and did not exhibit a prominent Th1 or Th2 cytokine bias in the inflamed mucosa. Similar results using the same PCR technique were obtained in a study carried out by German et al. (2000). However, the results obtained from Ridyard's (2002) investigation, indicated that lymphoplasmacytic colitis could be associated with an increased production of Th1 type cytokines (IL-2 and TNF-α) since they were increased in the colonic mucosa of affected dogs when compared to healthy controls. Ultimately, a series of studies (Peters et al. 2005; Maeda et al. 2011; Schmitz et al. 2012; Tamura et al. 2014) used the real-time reverse transcriptase PCR technique to make a similar assessment of the role of cytokine patterns in canine chronic enteropathies and in all of them the result was unanimous, with no predominance of one pattern over another. For this reason, to the authors' knowledge, in contrast to what happens in human inflammatory bowel disease, in veterinary medicine there seems to be no predominant cytokine profile in the inflamed intestinal mucosa of dogs, therefore, a Th1 or Th2 polarization has not been demonstrated.

In this project, it was not possible to conduct statistical analyses that would determine whether the difference in the expression of lymphocytes and plasma cells in the lamina propria was significant since this was not designed in the initial project. For this reason, during the histopathological evaluation of the samples, no cell counting was performed, impairing the identification of specific cell proportions.

3.5 – Limitations

The main limitation of this project was the reduced sample. Because the study stipulated that all participating animals had to be refractory to therapy for chronic enteropathy, a meticulous selection of animals that had successfully completed the disease's three main treatment pillars was necessary. In this search for animals that met these requirements, only eleven were found in the database of the Veterinary Teaching Hospital. Thus, in addition to a small sample, a very heterogeneous population both in terms of breeds and ages was also obtained.

The retrospective nature of the study is one of its limitations given that all data collection and clinical history are dependent on records written by different clinicians. In this study, all animals were followed by the Internal Medicine Service, which in part minimizes the number of clinicians writing consultation reports. Such reports include a summary of the animals' clinical history which lessens the possibility of losing crucial information. However, in some instances where these animals had routine consultations, or were admitted to emergency service due to gastrointestinal signs or even had to be hospitalized for several days, the assessment of the evolution of clinical signs used to be followed by different clinicians, which could have led to loss of information or a more subjective assessment of the severity of clinical signs and response to treatment demonstrated by different animals.

Another limitation of this study, which also stems from the fact that it is retrospective in nature, is the inability to calculate the CIBDAI or CCECAI activity indices. In this study, clinical signs were assessed before and after treatment, therefore, it would also have been essential to assess the evolution of the disease's severity before and after therapy in order to help clinicians and researchers assess the response to treatment and prognosis. This calculation requires the evaluation of parameters that were not continuously and clearly available throughout the animals' clinical record. To calculate these indices, it is necessary that this intention is expressed before collecting the animals' information so that a numerical classification can be assigned to a series of parameters that are generally only described verbally, such as the animals' attitude/activity, for example.

As was already mentioned, according to the histological evaluation of the intestinal samples, the lymphocytes and plasma cells in the lamina propria did not seem to be similarly distributed. However, as this was not the initial design of this study, at the time of histopathological evaluation of the samples, cell counting was not performed, thus statistical analysis that would have allowed the exploration of whether this difference was significant or not could not be conducted, which is another limitation of this project.

Finally, immunohistochemistry provides us with contemporary information regarding the presence or absence of the parasite in the intestine in relation to the lesions presented, which is essentially the main objective of this study. However, it would have been valuable to have a historical context of the animals with respect to Leishmania. Therefore, performing serology and PCR on hematopoietic organs such as the bone marrow of each animal would be beneficial to determine if the animal might have been previously infected with Leishmania or if it is currently infected but does not exhibit gastrointestinal leishmaniosis.

3.6 – Conclusion and Future Perspectives

The author's particular interest in gastroenterology combined with the possibility of completing the final curricular traineeship in the Internal Medicine Service prompted the choice of the subject of this master's thesis. This service offered daily contact with various chronic patients, many of which involved gastrointestinal disorders as they had a large daily caseload. The authors sought to establish a connection between refractory chronic enteropathy cases and leishmaniosis, especially in Lisbon, an endemic area. This was prompted by the presence of some more challenging treatment cases of RCE in this service and the rising number of reported cases in foreign countries where leishmaniosis only presented gastrointestinal clinical signs. To the authors' knowledge, no similar study had yet been done in Portugal.

Although *Leishmania* should still be considered as a differential diagnosis for chronic diarrhea, as supported by case reports of numerous authors, the negative immunohistochemistry results obtained in the project point to *Leishmania* as an unlikely causative agent in cases of refractory chronic enteropathy.

In a country where leishmaniosis is so prevalent, additional research on this subject at a much larger scale including animals with refractory chronic enteropathy from more than one hospital or clinic is required to determine the precise role of this protozoan in this disease. In future investigations, the ideal approach would be a prospective study with the sole purpose of investigating the relation between refractory chronic enteropathy and leishmaniosis. Conducting a prospective study instead of retrospective would facilitate better planning, overcoming some of the limitations inherent in this study. This design would allow assessment of clinical activity indices from initial consultation, before treatment, and could aim to ensure that, apart from emergencies, animals would be consistently examined by the same clinician. This would reduce subjectivity in assessment and minimize loss of information. This study would greatly benefit from both immunohistochemistry and PCR as diagnostic techniques, as these methods have proven to be complementary. PCR has a higher sensitivity making it more suitable for cases where the goal is solely to determine the positivity or negativity of a FFPE tissue sample, allowing the quantification of the parasitic load when performed quantitatively. However, in situations where there is interest in histological evaluation, as was in the present study where chronic inflammation was confirmed in each sample, or in cases where a correlation with histological lesions is desired (a scenario that is not possible by PCR), or in cases with economic restrictions, immunohistochemistry should be the initial step. In these specific cases, PCR can then serve as a complementary tool if a false negative immunohistochemistry is suspected, which, as reviewed, could arise due to limitations of the technique or low parasite loads not detectable by this approach.

Regardless of these limitations, this study opened new insights on the utility of immunohistochemistry for canine leishmaniosis in biopsy samples taken from dogs with refractory chronic enteropathy, questioning its current utility.

Despite being a less likely diagnosis, these results should not undermine the need to investigate cases of digestive *Leishmania*, particularly when accompanied by additional clinical or laboratory findings that support it, especially given the positive results reported in various articles published in other countries. Nevertheless, in refractory cases, it does not appear to be a causative agent.

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5 - Annexes

Annex 1 – Abstract for the Oral Presentation at ''*Encontro de Formação da Ordem dos Médicos Veterinários*''

OLD ENEMIES, NEW DIFFERENTIALS: CANINE LEISHMANIOSIS AS A CAUSE OF REFRACTORY ENTERITIS

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Refractory chronic enteropathy (RCE) accounts for a small but not insignificant percentage of cases in canine gastroenterology. Chronic diarrhea, albeit uncommon, is a clinical sign associated with canine leishmaniosis. Despite having already been explored in other countries, to the author's knowledge, a potential correlation between RCE and leishmaniosis has not yet been investigated in Portugal.

More than characterizing the histopathological profile of RCE, this study aims to explore a connection between RCE and leishmaniosis in dogs.

This retrospective study was conducted on cases of RCE undergoing gastroduodenoscopy and biopsy, between January of 2018 and October of 2022 at the Veterinary Teaching Hospital, University of Lisbon. Cases were reviewed for clinical signs, *Leishmania* serology screening and histopathology results. Immunohistochemistry for *Leishmania spp* was retrospectively performed in samples of all cases.

Eleven cases were included. All had diarrhea or loose stools and 9/11 (82%) presented weight loss. Serology for *Leishmania* was available for 5/11 (45%) and only 1/11 (20%) tested positive. From the total, 8/11 were gastroduodenoscopy samples while 6/11 were colonoscopy samples.

Most morphological changes were identified in the lamina propria and only 4/12 (33%) of small intestine (SI) samples and 2/6 (33%) of large intestine samples presented epithelial lesions. Overall, villous atrophy, villous fusion, crypt distension, mucosal fibrosis and lacteal dilation were more pronounced in the ileum than in the duodenum (100%, 75%, 75%, 75%, 100% versus 50%, 50%, 38%, 25%, 88%, respectively). The colon presented more marked fibrosis than the SI (83% versus 42%, respectively). Plasma cells predominated over lymphocytes in both the duodenum and the colon, while eosinophils and neutrophils were more frequently seen in the ileum. All samples were negative for *Leishmania* on immunohistochemistry.

These results highlight that plasma cells seem to be the predominant inflammatory population in RCE. Although *Leishmania* should still be included as a differential diagnosis of chronic diarrhea, it was not identified on immunohistochemistry in any of the cases, making it an unlikely causal agent in cases of RCE.

Annex 2 – Gastrointestinal Histopathology Standards (adapted from Day et al. 2008)

Histopathological standards for inflammation of the gastric body

Morphologic Criteria	Inflammatory Criteria
Surface epithelial injury	Intraepithelial lymphocytes
Gastric pit epithelial injury	Lamina propria lymphocytes and plasma cells
Fibrosis / Glandular nesting / Mucosal Atrophy	Lamina propria eosinophils
	Lamina propria neutrophils
	Gastric lymphofollicular hyperplasia

Histopathological standards for inflammation of the gastric antrum

Morphologic Criteria	Inflammatory Criteria
Epithelial injury	Intraepithelial lymphocytes
Epithelial hyperplasia	Lamina propria lymphocytes and plasma cells
Mucosal fibrosis / Glandular atrophy	Lamina propria eosinophils
	Lamina propria neutrophils
	Gastric lymphofollicular hyperplasia

Histopathological standards for inflammation of the duodenum

Morphologic Criteria	Inflammatory Criteria
Villous stunting	Canine intraepithelial lymphocytes
Villous epithelial injury	Feline intraepithelial lymphocytes
Crypt distension	Lamina propria lymphocytes and plasma cells
Lacteal dilation	Lamina propria eosinophils
Mucosal fibrosis	Lamina propria neutrophils

Histopathological standards for inflammation of the colon

Morphologic Criteria	Inflammatory Criteria
Surface epithelial injury	Lamina propria lymphocytes and plasma cells
Crypt hyperplasia	Superficial lamina propria eosinophils
Crypt dilation and distortion	Lamina propria neutrophils
Mucosal fibrosis and atrophy	Lamina propria macrophages

Annex 3 – Table Summarizing Results

Animal	Sex	Reproductive Status	Age at Diagnosis	Breed	Clinical Signs at Presentation	Refractory Clinical Signs	Laboratory Alterations*
1	Female	Spayed	11	Yorkshire Terrier	Unformed feces, Hematochezia, Mucus in the feces, Vomiting, Anorexia, Abdominal Spasm and Pain, Weight Loss.	Unformed feces, Hematochezia, Mucus in the feces, Vomiting, Anorexia, Weight Loss.	-
2	Male	Intact	7	Portuguese Water Dog	Diarrhea, Weight Loss.	Diarrhea, Hematochezia, Weight Loss.	Non-Regenerative Anemia, Leukocytosis, Neutrophilia, Hypoalbuminemia, Hypoproteinemia, Hypercobalaminemia.
3	Male	Unknown	3	Pomeranian	Diarrhea, Hematochezia.	Diarrhea, Hematochezia.	-
4	Female	Spayed	5	Golden Retriever	Vomiting, Diarrhea, Borborygmus, Anorexia, Weight Loss.	Vomiting, Diarrhea, Hematochezia, Weight Loss.	Non-Regenerative Anemia.
5	Male	Neutered	5	German Shepherd	Vomiting, Diarrhea, Weight Loss.	Diarrhea, Weight Loss.	Non-Regenerative Anemia, Hypocobalaminemia.
6	Female	Intact	3	Great Dane	Anorexia, Diarrhea, Weight Loss, Eructation.	Eructation, Nausea, Diarrhea.	Leukocytosis, Neutrophilia, Monocitosis, Thrombocytopenia, Hypoalbuminemia, Hypercobalaminemia.
7	Female	Spayed	7	Yorkshire Terrier	Unformed Feces, Vomiting, Abdominal Distension, Hyporexia.	Diarrhea, Mucus in the feces, Vomiting.	Leukocytosis, Neutrophilia, Thrombocytosis, Hypoalbuminemia, Hypoproteinemia.

*Conducted up to one month before endoscopy

Annex 3 Continuation – Table Summarizing Results

Animal	Sex	Reproductive Status	Age at Diagnosis	Breed	Clinical Signs at Presentation	Refractory Clinical Signs	Laboratory Alterations*
8	Male	Intact	1	Cross-breed	Vomiting, Diarrhea, Hematemesis, Hematochezia, Weight Loss.	Vomiting, Diarrhea, Hematochezia.	-
9	Male	Intact	9	Pinscher	Unformed Feces, Borborygmus, Abdominal Spasm and Pain, Weight Loss.	Abdominal Spasm and Pain, Borborygmus, Vomiting.	Increased Gamma-glutamyl transferase.
10	Male	Neutered	1	Boxer	Diarrhea, Weight Loss.	Diarrhea, Weight Loss.	Hypocobalaminemia.
11	Female	Spayed	8	Cross-breed	Diarrhea, Mucus in the feces, Vomiting, Weight Loss.	Vomiting, Diarrhea, Hematochezia.	Hypoalbuminemia, Hypoproteinemia, Increased concentration of urea, Increased Urinary protein-creatinine ratio.

*Conducted up to one month before endoscopy

Animal	Serology and PCR for *Leishmania*	Histopathological Evaluation	IHC for *Leishmania*	Diet Trial	Treatment Directed to Dysbiosis	Treatment with Immunosuppressants	Survival Time
1	Negative on Serology.	Chronic Inflammation.	Negative.	Hydrolyzed.	Metronidazole.	Prednisolone, Cyclosporine.	Lost to follow-up.
2	-	Chronic Inflammation.	Negative.	Homemade and then Highly Digestible.	Metronidazole, Enrofloxacin.	Prednisolone, Cyclosporine, Chlorambucil.	Euthanized
3	-	Chronic Inflammation.	Negative.	Hydrolyzed with Homemade.	Metronidazole, Probiotics.	Prednisolone.	Lost to follow-up.

PCR – Polymerase Chain Reaction; IHC – Immunohistochemistry.

Annex 3 Continuation – Table Summarizing Results

Animal	Serology and PCR for *Leishmania*	Histopathological Evaluation	IHC for *Leishmania*	Diet Trial	Treatment Directed to Dysbiosis	Treatment with Immunosuppressants	Survival Time
4	-	Chronic Inflammation.	Negative.	Hydrolyzed with Homemade.	Metronidazole, Amoxicillin with Clavulanic acid.	Prednisolone.	Unknown cause of death.
5	Negative on Serology.	Chronic Inflammation.	Negative.	Hydrolyzed, then Hydrolyzed with Highly Digestible and then Highly Digestible with Homemade.	Tylosin, Probiotics, Fecal transplantation.	Prednisolone.	Alive 1 year after diagnosis.
6	Positive on Serology and Negative on PCR from duodenum samples.	Chronic Inflammation.	Negative.	Homemade.	Metronidazole, Enrofloxacin, Probiotics.	Prednisolone.	Lost to follow-up.
7	Negative on Serology.	Chronic Inflammation.	Negative.	Hydrolyzed with Homemade.	Enrofloxacin, Probiotics.	Prednisolone, Cyclosporine.	Alive 3 years after diagnosis.
8	-	Chronic Inflammation.	Negative.	Hydrolyzed.	Metronidazole.	Prednisolone, Cyclosporine.	Alive 5 years after diagnosis.
9	-	Chronic Inflammation.	Negative.	Highly Digestible with Homemade.	Metronidazole.	Prednisolone.	Lost to follow-up.
10	-	Chronic Inflammation.	Negative.	Hydrolyzed.	Metronidazole.	Prednisolone.	Lost to follow-up.
11	Negative on Serology.	Chronic Inflammation.	Negative.	Hydrolyzed.	Metronidazole.	Prednisolone.	Euthanized.

PCR – Polymerase Chain Reaction; IHC – Immunohistochemistry.