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Veterinary Clinical Pathology: Case Studies in Diagnostic Practice

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Veterinary Clinical Pathology: Case Studies in Diagnostic Practice

Área científica: Patologia e Clínica Laboratorial

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ABSTRACT

This report reflects my internship at Cedivet – Laboratório Clínico Veterinário in the period from January 17th to May 8th 2024. It was conducted as a part of the Course Unit "Estágio" in the sixth year of my master's degree in veterinary medicine. My internship was mainly focused on the area of clinical pathology and its diagnostic methods.

Clinical pathology is a branch of pathology that dedicates itself to the use of laboratory methods to diagnose diseases. This includes a vast variety of diagnostic techniques such as cytology, haematology (including flow cytometric analysis and blood smear preparation and observation), biochemistry, coagulation tests, urinalysis, protein electrophoresis, and serology, among others. A big part of a pathologist's work comes from understanding each diagnostic exam, how it's done, how it can be interpreted and its limitations, to better understand possible artefact results. The sample's quality itself is also of major importance because it can lead to different interpretations and results of exams if not correctly stored and prepared. Therefore, pathologists have to make sure that all the results are valid and in line with the animal's clinical situation. This enables them to help clinicians and, together, reach a better and more accurate diagnosis and treatment.

During my internship at Cedivet, I had the opportunity to learn, practice and interpret each one of these techniques, mainly emphasizing on cytology and haematology exams. Histopathology and molecular biology cases were also part of my routine at the laboratory. In this document, three clinical cases are presented from a clinical pathology standpoint: leishmaniasis, immune-mediated haemolytic anaemia and feline lymphoma. For each one, several analyses were performed in order to reach a final diagnosis.

KEYWORDS: Leishmania; Anaemia; Lymphoma; Clinical Pathology

RESUMO

Este relatório reflete o meu estágio realizado na Cedivet – Laboratório Clínico Veterinário no período de 17 de janeiro a 8 de maio de 2024. Foi realizado no âmbito da Unidade Curricular "Estágio" pertencente ao 6º ano do Mestrado Integrado em Medicina Veterinária (MIMV). O meu estágio focou-se principalmente na área da patologia clínica e seus métodos de diagnóstico.

A patologia clínica é um ramo da patologia que se dedica ao diagnóstico de doenças, recorrendo a métodos laboratoriais. Estes incluem uma vasta gama de técnicas de diagnóstico como citologia, hematologia (incluindo análise por citometria de fluxo, bem como preparação e observação de esfregaço sanguíneo), provas bioquímicas e de coagulação, urianálise, eletroforese de proteínas, sorologia, entre outras. Uma grande parte do trabalho de um patologista vem de entender cada exame diagnóstico, como é feito, como é interpretado e as tuas limitações, para conseguir perceber possíveis resultados artefactuais. A qualidade da amostra é de elevada importância pois pode levar a diferentes interpretações e resultados de exames, caso não seja corretamente armazenada e preparada. Portanto, os patologistas têm de garantir que todos os resultados são válidos e concordantes com a situação clínica do animal. Tal facto permite-lhes auxiliar os clínicos e, juntos, chegar a um melhor e mais preciso diagnóstico e tratamento.

Durante o meu estágio na Cedivet, tive a oportunidade de experienciar cada um destes campos, acabando por dar mais enfâse à citologia e hematologia. Casos de histopatologia e biologia molecular também fizeram parte da minha rotina no laboratório. Para este relatório, são apresentados três casos clínicos do ponto de vista da patologia clínica: leishmaniose, anemia hemolítica de mediação imune e linfoma felino. Para cada um, várias análises foram realizadas para a conclusão do diagnóstico final.

PALAVRAS-CHAVE: Leishmania; Anemia; Linfoma; Patologia Clínica

CASE-LOG

In this chapter, it is presented the case-log during my internship at Cedivet in the period from January 17th to May 8th, 2024. Cases are distributed on different tables and sorted by area/diagnostic method and species. Important to note that, for diagnostic purposes, for the same animal more than one complementary exam was perfomed. Also to mention that the vast majority total haematology cases had a blood smear analysis. Cases involving immunocytochemistry and immunohistochemistry were also analysed.

Cytology

Organ / ***Sample***	**Animal species**			**Total**
	Dog	**Cat**	**Hedgehog**	
Skin/Subcutaneous Tissue (nodules)	89	34	0	123
Liver	7	3	0	10
Spleen	7	4	0	11
Mammary Gland	4	6	1	11
Ear canal	3	0	0	3
Lymph nodes	16	5	0	21
Pleural effusions	1	5	0	6
Abdominal effusions	2	0	0	2
Cystic fluids (dermal)	3	1	0	4
Cerebrospinal fluid	6	2	0	8
Bone marrow	6	1	0	7
Intestine	2	0	0	2
Lung	1	0	0	1
Kidney	0	3	0	3
Adrenal	1	0	0	0
Prostate	4	0	0	4
Bladder	2	2	0	4
Vagina	1	0	0	1
Intra-abdominal/throracic mass	3	0	0	3
Bone	2	1	0	3

Haematology (total cases)

Dog	96
Cat	79
Equine	1
Others	6

**Others – lhama and bird*

Haematology (others)

Blood Smears	110-146
SAT	12
DAT	5
Bone Marrow analysis	3

Histopathology

Organ	**Dog**	**Cat**	**Total**
Skin / Subcutaneous Tissue	18	7	25
Mammary Gland	16	13	29
Oral cavity	12	1	13
Spleen	6	0	6
Eye	2	0	2
Testicle	5	0	5
Intestine	3	2	5
Bone	4	0	4
Bladder	3	1	4
Ear canal	5	0	5
Lymph nodes	10	3	13
Nasal cavity	0	2	2

Urinalysis/Sediment

Dog	21
Cat	32

Protein Electrophoresis

Dog	20
Cat	16
Parrot	1

Other Exams

Coprology	5
Biochemistry/Endocrinology/Coagulation	146
Serology	8
PCR	23

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Agradeço, primeiramente à Professora Doutora Irina Amorim por toda a ajuda, compreensão, profissionalismo durante este ano. Desde o primeiro momento, revelou total disponibilidade para esclarecer todas dúvidas, proporcionando-me liberdade e segurança durante este ano de conclusão do mestrado.

A toda a equipa da Cedivet – Laboratório Veterinário agradeço por tudo aquilo que me ensinaram ao longo do meu estágio. Em especial ao Dr. Hugo Carvalho por me ter dado a oportunidade de desenvolver o meu trabalho nesta prezada instituição e, também, por toda a simpatia e disponibilidade.

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A todos os meus amigos um grande obrigado por terem feito parte do meu caminho e terem feito estes anos valerem a pena. Agradeço por todas as memórias que criamos juntos e que certamente nunca esquecerei.

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Abbreviations

ALP Alkaline phosphatase

APP Acute phase protein

APTT Prolonged activated partial thromboplastin time

ATIII Anti-thrombin III

CDR3 Complementary determining region

Chol Cholesterol

DAT Antiglobulin Direct Test

DIC Disseminated intravascular coagulation

ELISA Enzyme-Linked Immunosorbent Assay

FC Flow cytometry

FeLV Feline leukaemia virus

FIV Feline immunodeficiency virus

FNA Fine needle aspiration

FNNA Non-aspirate fine needle

GFR Glomerular filtration rate

GGT Gamma-glutamyl transferase

GI Gastrointestinal

H&E Haematoxylin and eosin

Hb Haemoglobin concentration

Hct Haematocrit

I/HGAL Intermediate or high-grade alimentary lymphoma

ICT Immunochromatographic tests

IFAT Immunofluorescence Antibody Test

IMHA Immune-mediated haemolytic anaemia

IMT Immune-mediated thrombocytopenia

LGAL Low-grade alimentary lymphoma

LGL Large granular lymphoma

MAC Complement membrane attack complex

MCHC Mean corpuscular haemoglobin concentration

MGG May-Grunwald Giemsa

MRD Minimal residual disease

NO Nitric oxide

PARR PCR assay for antigen receptor rearrangement

PCR Polymerase chain reaction

PCV Packed Cell Volume

PT Prolonged prothrombin time

PTE Pulmonary thromboembolism

qPCR Quantitative PCR

RBC Red blood cell

RT-qPCR Reverse transcriptase quantitative PCR

SAT Saline Agglutination test

SDMA Symmetric dimethylarginine

TG Triglycerides

TLRs Toll-like receptor

1. LEISHMANIASIS

1.1 Introduction

Leishmaniasis, also known as leishmaniosis, is one of the most relevant conditions in veterinary medicine, responsible for causing multisystemic disease in dogs, cats, horses, and other mammals - including humans - therefore being an agent of high zoonotic, animal health and public health importance for which accurate diagnosis is paramount (Nelson & Couto, 2021).

The agent responsible for its transmission is the protozoan *Leishmania spp.,* with over 20 species being known (WHO, 2023). It is endemic in the Mediterranean basin, Middle East, and South and Central America. Despite the fact many species are known, we shall be focusing only on *Leishmania infantum* for the purpose of this chapter, since this is the most relevant reported in Portugal. A national survey conducted in 2022 (for a better understanding of continental Portugal's status on leishmaniasis) showed a seroprevalence of 12. 5% in a sample of 1086 dogs. The same study also concluded that the districts of Castelo Branco, Guarda, and Portalegre were the most affected by this problem (Almeida et al., 2022).

Leishmania is, therefore, an important and contemporary topic in the veterinary field in Portugal (Almeida et al., 2022).

1.2 Life cycle and biology

L. infantum relies heavily on vectors for its transmission to mammalian hosts such as dogs (which also serve as main reservoirs). These vectors are usually sandflies from the *Phlebotomus* species, and are mostly active from April to October, making these months crucial in the transmission process - understanding the activity patterns of these vectors is essential for effective preventive medicine. Note that, due to climate change, this seasonality has become more volatile and vectors are currently expanding both on time and space frame, being present throughout the year and also expanding to new geographic areas of the globe. Despite sandflies being the most common vectors, there are also reports of ticks and fleas acting as vectors in the leishmaniasis life cycle (Ribeiro et al., 2018; Semenza & Paz, 2021).

When female sandflies feed on healthy dogs (through a bite and a blood meal) they pass along the promastigote form of the parasite, thus infecting the animal. The promastigote is then phagocytized by the host´s macrophages, turning into the amastigote form, which starts multiplying within the cell. Eventually, the cell´s cytoplasmatic membrane will rupture and amastigotes will spread through the bloodstream, invading mononuclear phagocytes from several organs (especially the liver, spleen, lymph nodes and bone marrow). Once another

female sandfly takes a new blood meal of an already diseased dog, it will ingest the phagocytic cells infected with amastigote forms which then return to the promastigote stage once they reach the insect's digestive system. It's important to notice that promastigote forms are capable of mobility, having a flagellum that allows them to migrate from the insect´s gut to its mouth parts and be ready to infect upon a blood meal, continuing the cycle. The amastigote forms are not mobile, thus needing to be phagocytised. Other mammals, including humans (as mentioned) and even cats (though less commonly than dogs) can also host this protozoan leading to illness (Morales-Yuste et al., 2022).

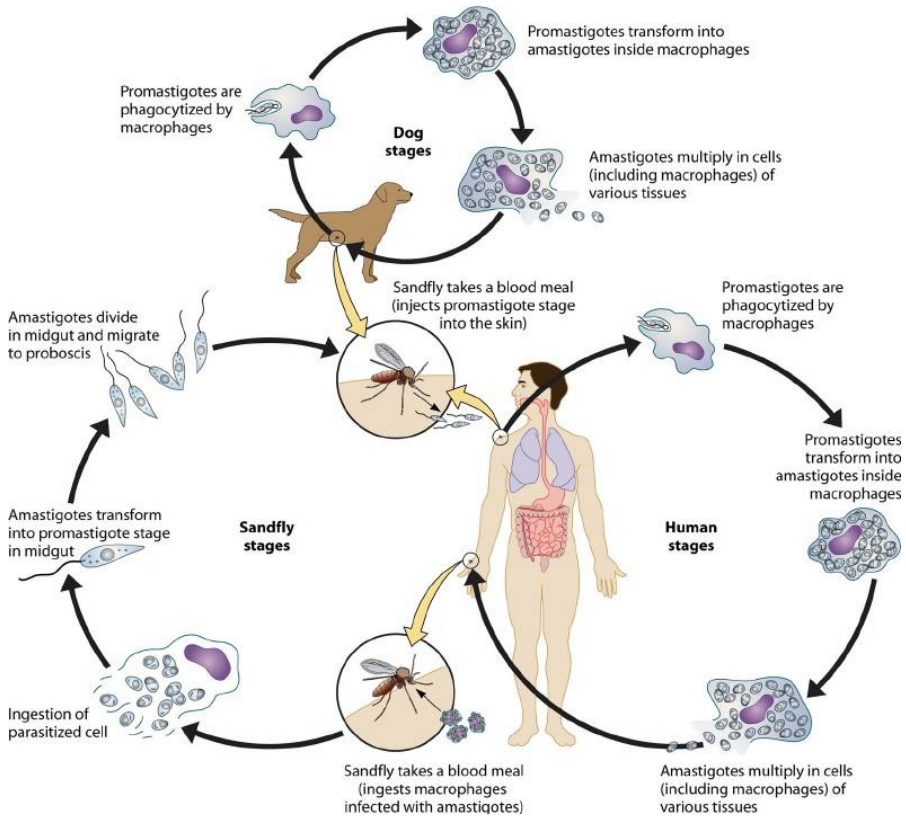


Figure 1- Leishmania spp. life cycle. Adapted from Esch & Petersen, 2013.

1.3 Pathogenesis and clinical signs

Once the macrophages are infected with promastigote forms, these parasites transform into the amastigote forms. The transformation occurs inside the macrophagic cell´s vacuoles avoiding its signalling pathways and allowing the then amastigote forms to multiply until they burst out of the mononuclear cell, resulting in their release into the bloodstream, leading to the infection of other phagocytic-type cells from the blood, liver, spleen, and bone marrow (Morales-Yuste M. 2022).

The pathogenesis behind *L. infantum* infection lies within an unbalanced host immune and cellular response. If the response is mainly cellular, Th1 cytokines (IFN-gamma and TNF-alpha) will stimulate macrophages to produce nitric oxide (NO) and other molecules to eliminate

the parasite. On the other hand, if the response is mainly humoral, Th2 cytokines (IL-10, IL-13, IL-4, and TGF-beta) will stimulate the production of immunoglobulins leading to the inactivation of the macrophagic response. Therefore, a cellular response will result more likely in the elimination of the disease while a humoral response will more likely make the disease progress. The pathogenic mechanisms are still not fully understood and there is evidence of molecules like the toll-like receptors (TLRs) being involved in the progression of leishmaniosis (Morales-Yuste M. 2022).

Leishmaniasis can be expressed as a cutaneous and/or visceral presentation. Cutaneous lesions are very common and can include several forms of dermatitis with or without alopecia and pruritus, nasal hyperkeratosis, depigmentation, and onychogryphosis. This form is also often accompanied by lymphadenopathy from the popliteal, prescapular, and submaxillary lymph nodes, which usually appear earlier than the cutaneous lesions (Ribeiro et al., 2018).

In the visceral form, the most affected organs are the spleen, liver, kidney, and bone marrow. The spleen usually carries a higher parasitic load presenting with hypertrophy and hyperplasia of the red pulp and infiltration of mononuclear and plasma cells. In the white pulp, lymphocytes substitute macrophages. The liver is also severely affected by *Leishmania infantum* through its Kupffer cells (mononuclear phagocytic cells resident in this organ) leading to granuloma formation, because of the inability to eliminate the parasite (Koutinas & Koutinas, 2014).

In the kidneys of infected animals, glomerulonephritis due to immune complex deposition is the most relevant clinical sign and one of the most important because it often leads to death by renal failure (Parody et al., 2019).

When the bone marrow is affected, changes occur in hematopoietic cell production, leading to pancytopenia, non-regenerative anaemia, histiocytic hyperplasia, erythrocytic hypoplasia, and medullar aplasia (Morales-Yuste M. 2022).

Other signs can be poor body condition, ocular damage (blepharitis, uveitis, and conjunctivitis), and coagulation problems (Morales-Yuste et al., 2022).

1.4 Diagnosis

The diagnosis of leishmaniasis can be achieved by combining clinical signs with laboratory tests and findings. The testing can be direct (through the visualization of the parasite) or indirect.

Three major forms of laboratory diagnosis can be used (Paltrinieri et al., 2016):

- Cytology/histopathology
- Molecular biology - Polymerase chain reaction (PCR);

- Serology - Immunofluorescence Antibody Test (IFAT) and Enzyme-Linked Immunosorbent Assay (ELISA).

The first two are direct methods of diagnosis since we directly detect the pathogen's presence, while the last one is indirect since we evaluate its presence by analysing the immune response against it (antibody titles).

Cytology focuses on finding the amastigote forms (free or within macrophages) which are usually accompanied by a macrophagic, neutrophilic and lymphoplasmocytic infiltrate. The best samples depend on the clinical picture (Valenciano & Cowell, 2020):

- If cutaneous lesions are found, these should be aspirated;
- If there's lymphadenopathy, lymph nodes can be sampled;
- If hepatomegaly or splenomegaly or nodular lesions are found within these organs, they should be aspirated;
- If haematology is consistent, a bone marrow sample can be useful.

Overall, cytology is a good and quick method of diagnosis and can also be used in fluids such as synovial or cerebrospinal fluid and effusions in suspected animals. The finding of just one amastigote form is enough to consolidate diagnosis (Ribeiro et al., 2018).

Histopathology can be performed in a great variety of tissues allowing pathologists to observe the same type of infiltrate as the one mentioned above, plus the cytoarchitecture of samples (Paltrinieri et al., 2016; Pinto et al., 2011). Important to note that both complementary exams need a relatively high parasitic load sample for a conclusive analysis. In cases where the parasite is not clearly identified but the lesion pattern and clinical signs are suggestive, PCR or serology should be considered. (Paltrinieri et al., 2016). Some authors also refer the use of immunohistochemistry with polyclonal rabbit anti-*Leishmania* antibody as another alternative for inconclusive cases (Casanova et al., 2019).

Molecular biology through PCR presents a higher sensitivity than the two methods above mentioned. It has the advantage of being able to be performed in a wide range of samples such as actual tissue or biological fluid. It can even be done on already prepared cytological or paraffin-embedded material (Paltrinieri et al., 2016). Routinely, the available options are conventional PCR, nested PCR, and quantitative (real-time) PCR that target subunits of ribosomal RNA genes and kinetoplast DNA minicircles. Notably, real-time PCR can be useful while monitoring an infected animal since it can quantify protozoan DNA before and after treatment (Galluzzi et al., 2018)

Finally, with serology, two main tests are usually offered in veterinary laboratories: IFAT (gold standard) and ELISA. These tests can detect and quantify the presence of antibodies against the parasite, for which we can use the dog´s plasma or serum, each method requiring a

different approach. In brief, IFAT relies on the fluorescence emitted by the fluorochrome following the antibody-antigen binding in the sample, whereas ELISA bases itself on the colour change of the well after the same binding, associated with a substrate. Both IFAT and ELISA involve successive dilutions to determine the point of positivity in the sample and thereby infer the concentration of specific immunoglobulins. However, despite these two tests exhibiting high sensitivity and specificity, simpler protocols are done routinely by clinicians using immunochromatographic tests (ICT). The ICTs are only qualitative and depend a lot on the clinical stage of the animal. The biggest obstacle of leishmaniasis serology is when it comes to vaccinated dogs because there is still no viable way to distinguish naturally formed antibodies from those derived from vaccination. Additionally, IFAT and ELISA manufacturers of tests have different cut-offs of positivity and the result can also be influenced by the operator´s experience (Paltrinieri et al., 2016).

1.5 Clinical Pathology findings

1.5.1 Haematology

The two most relevant laboratory findings in *L. infantum* infected dogs are mild to moderate normocytic normochromic anaemia and a hypercoagulable state, usually derived from chronic disease. Leishmaniasis-resulting renal failure leads to reduced erythropoietin production by the kidneys affecting erythroid lineages, justifying the anaemia. The haemostatic abnormality comes from protein-losing nephropathy and subsequent loss of anti-thrombin III (ATIII) which is an important anticoagulant protein. This is capable of preventing the conversion of fibrinogen into fibrin molecules and, once reduced, induces thrombosis and consumption coagulopathies (Paltrinieri et al., 2016).

1.5.2 Biochemistry

The most relevant biochemical findings relate to renal function and the chronic kidney disease they tend to develop. Under this topic, it is of great importance to monitor its filtrating capability through biomarkers (Paltrinieri et al., 2016).

In recent years, symmetric dimethylarginine (SDMA) has shown to be a good indicator of decreased glomerular filtration rate (GFR). It can increase up to 40% with just 25% of kidney function lost, therefore clinicians can better predict and handle cases. (Hall et al., 2016). Proteinuria is a mandatory aspect to monitor in Leishmania-infected animals using methods primarily like the dipstick and then, if further evidence is needed, the urinary protein-to-

creatinine ratio (UPC) may be determined for better staging of the patient (Paltrinieri et al., 2016).

Hepatic and pancreatic inflammation findings can occur depending on what form of the disease the patient has and other comorbidities (Morales-Yuste et al., 2022)

1.5.3 Protein electrophoresis

Due to the nature of this disease (pro-inflammatory state), dogs afflicted with leishmaniasis are expected to have their total proteins and globulins increased (hyperproteinaemia by hyperglobulinemia, or, in some cases/stages of the disease, only hyperglobulinemia). Because of this, protein electrophoresis is useful not only when it comes to quantification but also in the evaluation of the resulting graph (the proteinogram). This technique allows an evaluation of the migration of a sample´s proteins when submitted to an electric force. These macromolecules are then separated by size and charge. Samples used are mostly serum or plasma, but some authors do not recommend plasma because it has fibrinogen which migrates to the beta and gamma globulin zones which can lead to a confusing pattern that isn't so easy to read, due to the overlap of fractions. With serum protein electrophoresis we usually are able to separate four to five fractions: Albumin, alpha-globulins (can be split in alpha-1 and alpha-2), beta-globulins and gamma-globulins. These last two fractions are usually increased in cases of chronic illnesses and neoplasia, showing different electrophoretic patterns (we can have the same order of values but a different proteinogram). So, the observation of different protein fractions allows a quick interpretation of results (Marques, 2014)

In pro-inflammatory states, albumin (a negative inflammatory marker) is expected to decrease, not only because it's a negative acute phase protein (APP) but also because, in many cases, animals suffer from proteinuric nephropathy, losing it in urine. Another important reason is oncotic pressure balance: If globulins increase, this may favour the loss of albumin in order to maintain blood pressure within adequate ranges (Gounden et al., 2023). Consequently, the Albumin: Globulin (A: G) ratio will also decrease. Both hypoalbuminemia and a low A: G ratio are bad prognostic factors. When it comes to globulins, beta and gamma globulins are usually increased with Leishmaniasis (Paltrinieri et al., 2016). These physiological alterations result in a proteinogram with decreased albumin and a polyclonal pattern with augmented beta and gamma fractions. It is important to emphasize that this kind of graphic presentation is characteristic of chronic processes and not pathognomonic of leishmaniasis (Marques, 2014). But, in the context of an infected animal, it is usually the main reason for a polyclonal gammopathy and can be also used to monitor the clinical response, combined with clinical signs.

In conclusion, protein electrophoresis is a good form of monitoring patients through their protein profile since, with treatment, animals will normalise albumin and globulin values. (Marques, 2014; Paltrinieri et al., 2016).

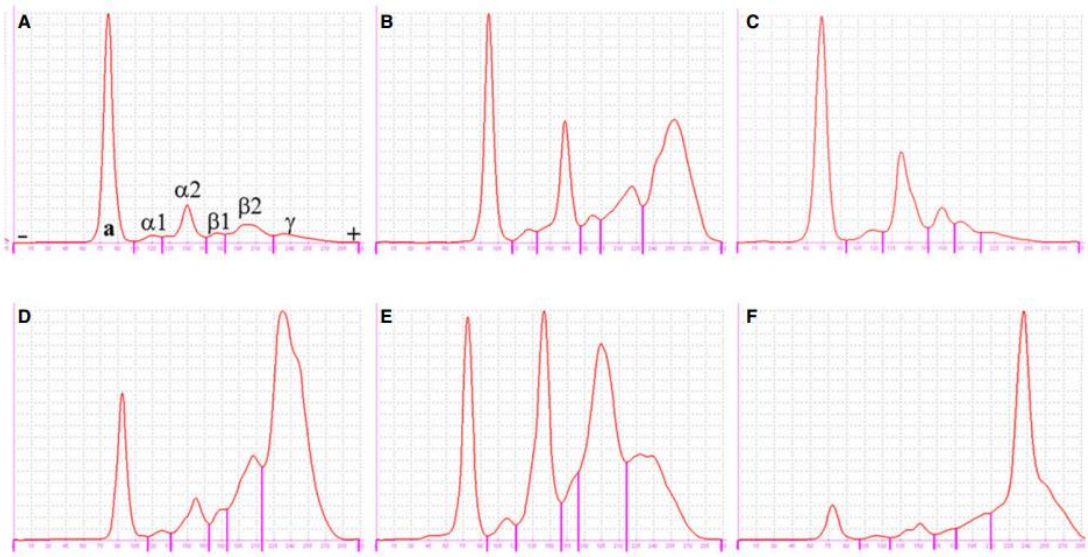


Figure 2 - Protein Electrophoresis patterns in dogs. (A) Normal. (B-F) Polycolonal patterns possible. Alpha-2 fraction can relate to an earlier, more acute, stage of leishmaniasis. Oligoclonal peak in (F) can be due to other chronic comorbidities. Adaptated from Paltrinieri et al., 2016.

1.6 **Clinical Case: Zimba** (reference intervals present in attachments)

Patient: Zimba is a 7-year-old Rhodesian Ridgeback male, presenting with internal widespread ulcerated lesions throughout the gastrointestinal tract (GIT) from the oral mucosa to the large intestine.

- **21/01/2024**

Exams performed: Tissue samples of the oral mucosa and intestine (small and large) were collected, sent for histopathological evaluation and routinely processed. Stain: Haematoxylin and Eosin (H&E).

Results: Microscopically, the oral mucosa tissue presented a focally extensive mixed inflammatory infiltrate. It was essentially composed of an intense population of activated macrophages, neutrophils and a smaller amount of lymphocytes.

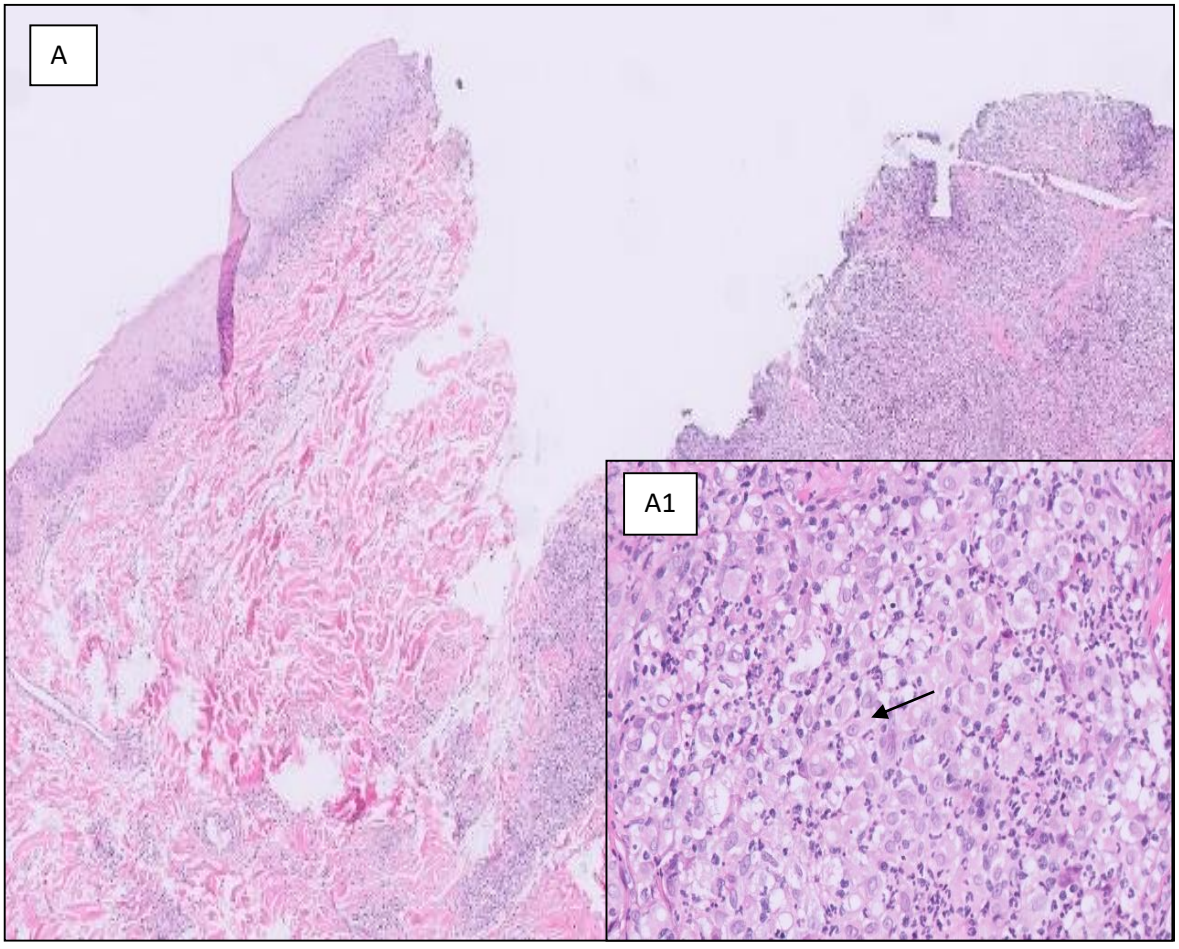
The intestinal samples were majorly ulcerated. A large number of round cells with histiocytic appearance, resembling the ones present on the oral mucosa, were predominant in the lamina propria. Neutrophils and a smaller number of lymphocytes were also observed. Moderate fibrosis was present on the *lamina propria* as well as a shortening of intestinal *villi* and ectasia of lymphatic ductes.

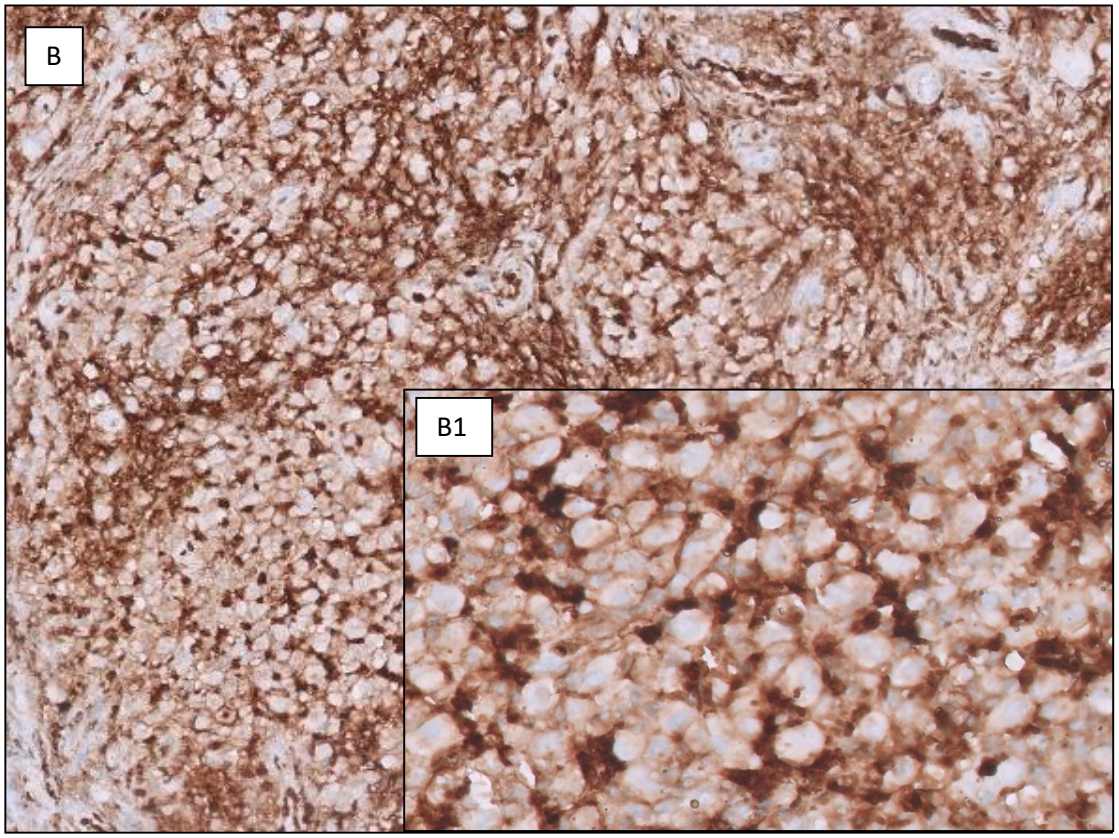
Mild atypia was present in the inflammatory population and no mitotic figures were observed in both samples.

To better conclude about the presence of microorganisms *Periodic Shiff-Acid*, *Gram*, and *Fite-Faraco* were requested, all of which resulted negative. Immunohistochemistry was also performed with anti-CD18 (histiocytic cell marker) and anti-CD117 (mast cell marker) to assess the true origin of the predominant round cell population. Results showed anti-CD18 being positive for more than 90% of the cells, reinforcing their histiocytic origin and thus confirming the inflammatory nature of the process.

Diagnosis: Piogranulomatous stomatitis and enteritis.

Observations: The predominantly histiocytic and neutrophilic infiltrate, with mild atypia, revealed a pyogranulomatous multifocal infiltrate. This kind of infiltrate tends to be non-specific in aetiology being possible in, for example, the infectious process of several agents. Neoplastic processes of histiocytic origin could be differentials to consider but seemed less likely due to the mild atypia that cells had.





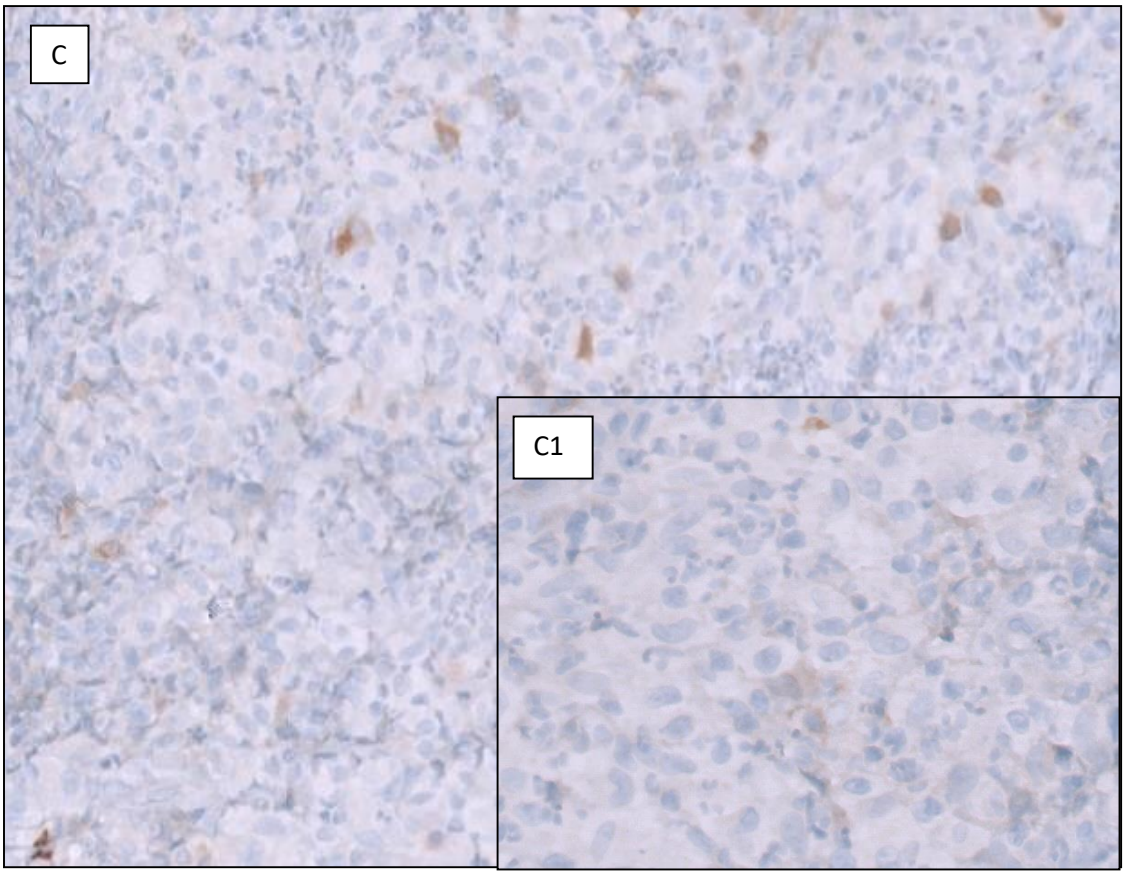


Figure 3 - Samples for Zimba´s oral mucosa. A) Tissue ulceration and focally extensive mixed inflammatory infiltrate. Hematoxylin and eosin (H&E). 2x. A1) Mixed inflammatory infiltrate present in the oral mucosa mainly composed of activated macrophages (black arrow), neutrophils and a smaller amount of lymphocytes. Hematoxylin and eosin (H&E). 20x. B) Immunohistochemistry with anti-CD18. Immunoperoxidase-diaminobenzidine stain with Mayer's haematoxylin counterstain. 20x. B1) Inset shows strong membranous CD18 immunoreactivity compatible with macrophages. Immunoperoxidase-diaminobenzidine stain with Mayer's haematoxylin counterstain. 40x. C) Immunohistochemistry with anti-CD117. Immunoperoxidase-diaminobenzidine stain with Mayer's haematoxylin counterstain.20x. C1) The great majority of the inflammatory cells were CD117 immunonegative, excluding their potential mast cell histogenesis. Immunoperoxidase-diaminobenzidine stain with Mayer's haematoxylin counterstain. 40x.

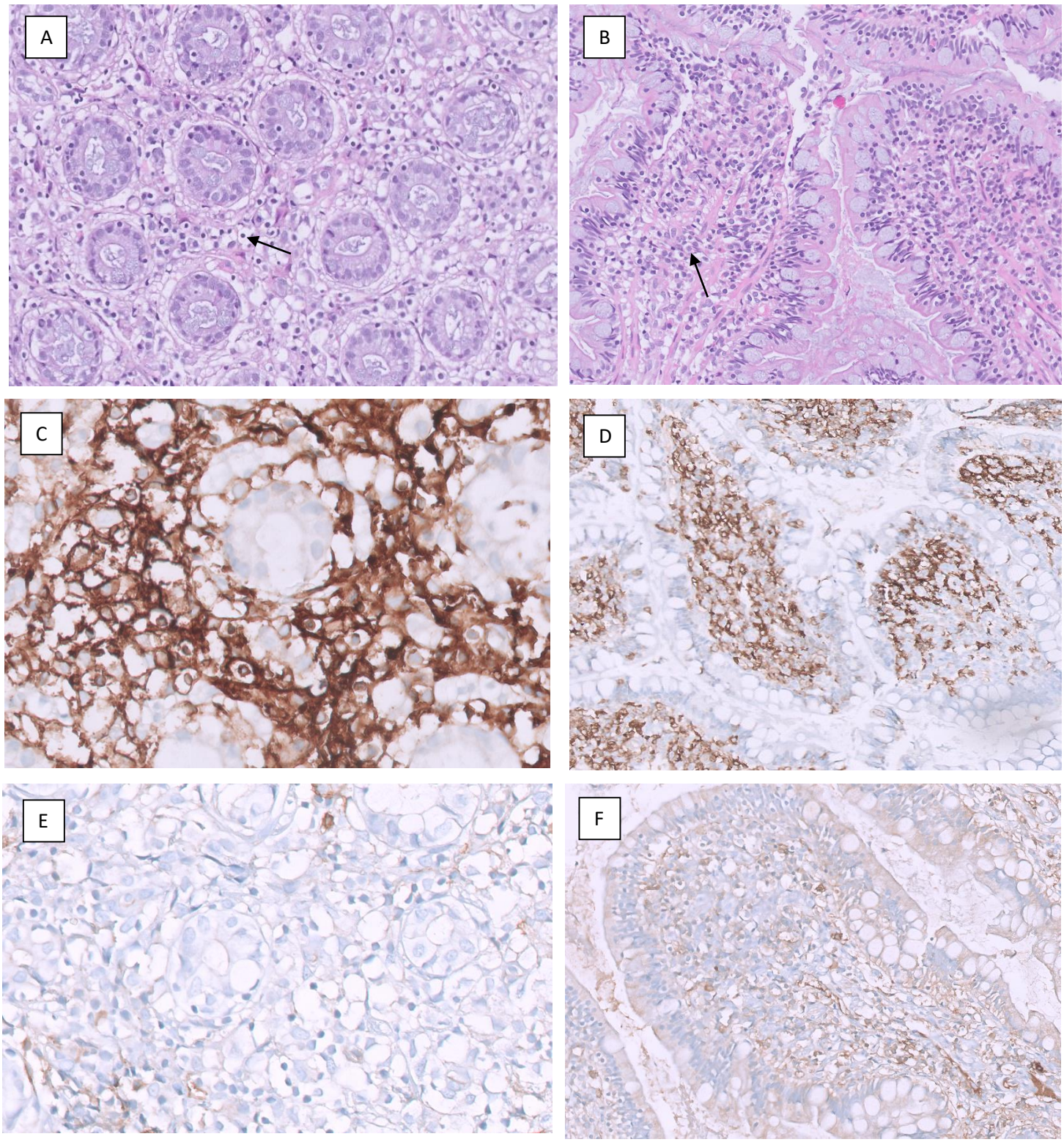


Figure 4 - Microscopic evaluation of Zimba´s intestinal biopsy samples. A) The lamina propria of duodenum is heavily infiltrated with severe and diffuse mixed inflammatory infiltrate, consisting mainly of macrophages (black arrow) and scattered neutrophils and lymphocytes. Hematoxylin and eosin (H&E). 20x. B) Note the inflammatory infiltrate, mainly macrophages (black arrow), in the superficial lamina propria of the cript. Hematoxylin and eosin (H&E). 20x. C) Inset shows strong membranous CD18 immunoreactivity compatible with macrophages. Immunoperoxidase-diaminobenzidine stain with Mayer's haematoxylin counterstain. 40x. D) Immunohistochemistry with anti-CD18. Immunoperoxidase-diaminobenzidine stain with Mayer's haematoxylin counterstain. 20x. E) The great majority of the inflammatory cells were CD117 immunonegative, excluding their potential mast cell histogenesis.Immunoperoxidase-diaminobenzidine stain with Mayer's haematoxylin counterstain. 40x. F) Immunohistochemistry with anti-CD117. Immunoperoxidase-diaminobenzidine stain with Mayer's haematoxylin counterstain. 20x.

- **22/02/2024**

During a re-evaluation, an ultrasound was performed and splenomegaly was observed.

Exam performed: Ultrasound-guided fine needle aspiration cytology (FNA) and fine needle non-aspiration cytology (FNNA) of splenic lesions. Stain: May-Grunwald Giemsa (MGG)

Results: Microscopically**,** activated macrophages were observed, presenting phagocytosis of oval-like structures compatible with amastigote forms of *Leishmania* spp.. These were also present outside of cells. Neutrophils and some lymphocytes were also observed.

Diagnosis: Pyogranulomatous inflammation associated with *Leishmania* spp.

Observations: Given this diagnosis, PCR on the previous histological blocks was suggested to determine if *Leishmania spp.* could also be present in the other widespread lesions.

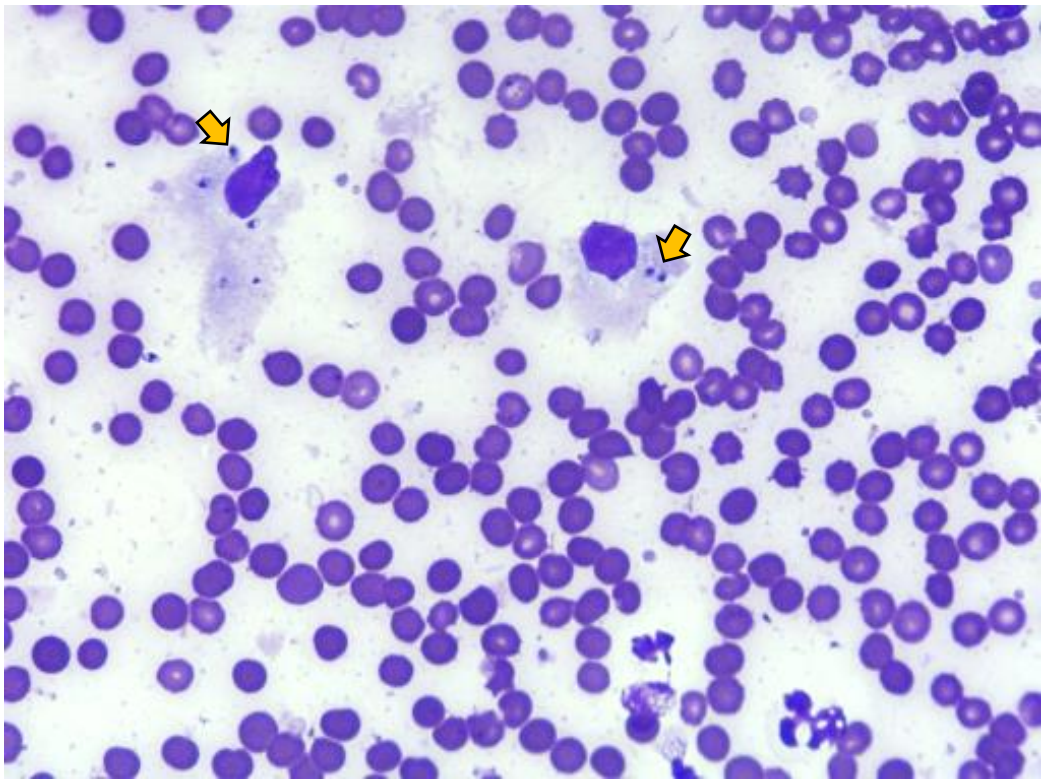


Figure 5 - Fine needle aspiration of the nodules present on Zimba´s spleen showing Leishmania spp. amastigotes (gold arrow) inside macrophages. May-Grunwald Giemsa (MGG). 40x

- **23/02/2024**

Exam performed: *TaqMan* RT-qPCR using DNA extracted from paraffin-blocks.

Result: Positive for *Leishmania spp.*

Leishmania spp. (PCR)	**Detetado**
Amostra: Bloco histológico.	

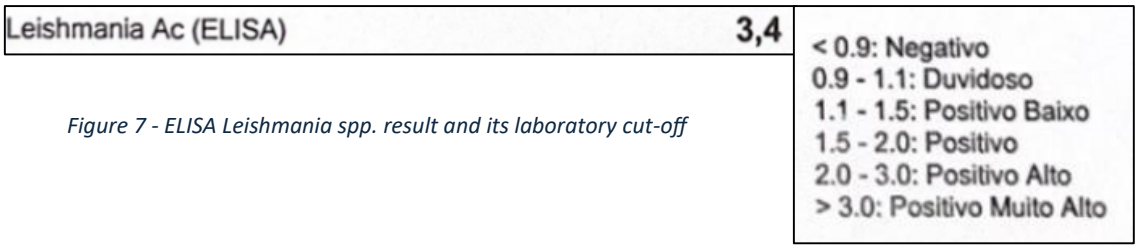
Figure 6 - Leishmania spp. PCR result

- **28/02/2024**

Exam performed: Protein Electrophoresis and ELISA.

Results: Protein Electrophoresis (Capillary Zone) revealed a decrease in Albumin. Despite normal total values in the globulin fractions, they were a visible elevation of Alpha-2, Beta, and Gamma globulins, compatible with a polyclonal tracing. The *Leishmania spp.* ELISA was 3.4.

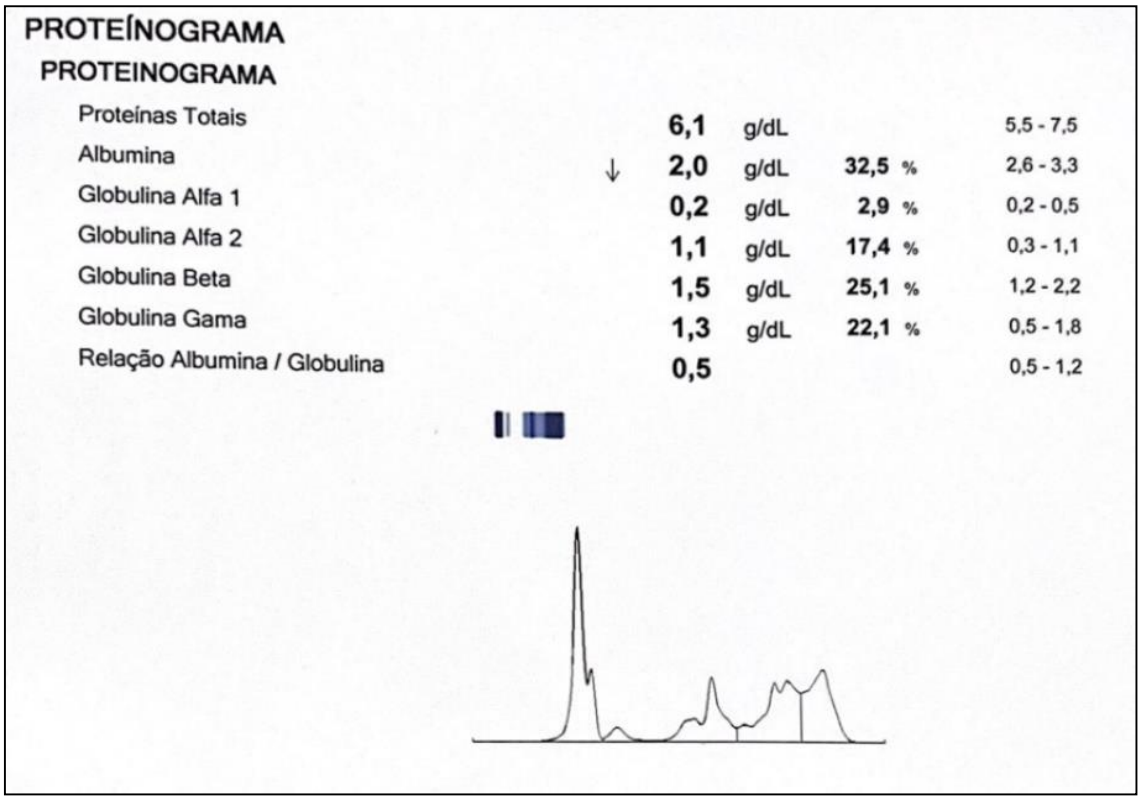
Diagnosis: The polyclonal pattern present in the protein electrophoresis can be justified by leishmaniasis. When it comes to the ELISA, a 3.4 value equals a highly positive sample for *Leishmania spp* antibodies.



Leishmania Ac (ELISA)	3,4

< 0.9: Negativo
0.9 - 1.1: Duvidoso
1.1 - 1.5: Positivo Baixo
1.5 - 2.0: Positivo
2.0 - 3.0: Positivo Alto
> 3.0: Positivo Muito Alto

Figure 7 - ELISA Leishmania spp. result and its laboratory cut-off



PROTEÍNOGRAMA

PROTEINOGRAMA

Proteínas Totais	6,1	g/dL		5,5 - 7,5
Albumina	↓ 2,0	g/dL	32,5 %	2,6 - 3,3
Globulina Alfa 1	0,2	g/dL	2,9 %	0,2 - 0,5
Globulina Alfa 2	1,1	g/dL	17,4 %	0,3 - 1,1
Globulina Beta	1,5	g/dL	25,1 %	1,2 - 2,2
Globulina Gama	1,3	g/dL	22,1 %	0,5 - 1,8
Relação Albumina / Globulina	0,5			0,5 - 1,2

Figure 8 - Proteinogram Leishmania spp. result and its laboratory cut-off

1.7 Discussion & the role of Clinical Pathology

In Zimba´s case, several methods were considered for the final diagnosis of infection by *Leishmania infatum*.

The histopathological exam revealed a mixed inflammatory infiltrate mainly constituted by activated macrophages but also with neutrophils and lymphocytes. At the time of diagnosis, a pyogranulomatous process was considered most likely and *Gram, Fite-Faraco* and *Periodic Schiff-Acid* stains were requested to assess for infectious agents and were all negative. *Gram* tends to stain gram-positive bacteria, *PAS* tends to stain glycogen and fungus and *Fite-Faraco* tends to stain acid-fast bacteria (Sharkey et al., 2020). None of these markers are used to identify *Leishmania* spp., but was important to rule out other differentials and reach the final diagnosis. Immunohistochemistry was done as well with antibodies anti-CD18 and anti-CD117, being positive in more than 90% of cells for anti-CD18. This was specifically to distinguish if the round cells observed in the sample were from histiocytic origin (anti-CD18 marker) or mast cells (anti-CD117 marker). In regards to this first analysis, the type of infiltrate described is considered typical of leishmaniasis (Koutinas & Koutinas, 2014) but not pathognomonic as it can result from other agent´s infection. Immunohistochemistry with e.g. *streptoavidin peroxidase* (Pinto et al., 2011) or polyclonal rabbit anti-*Leishmania* antibody (Casanova et al., 2019) could have been done to better identify *Leishmania spp.* amastigotes or even PCR analysis, though both authors emphasise that these methods of diagnosis may fail if the sample parasitic load is low (Paltrinieri et al., 2016).

A month later, spleen cytology was performed due to splenomegaly observed by ultrasound. Amastigotes were described inside the spleen´s macrophages along with an inflammatory infiltrate similar to that previously reported in the histopathological exam of the intestinal samples. The spleen is one of the main organs for this parasite to be found, cytology is reported as an easy method of diagnosing it through its observation (Koutinas & Koutinas, 2014) and the cytological image was compatible with the diagnosis. As in histopathology, cytology also relies on a good parasitic load on its samples for a good diagnosis, which is the principal disadvantage of both methods (Paltrinieri et al., 2016).

This finding led to DNA extraction from the paraffin block for PCR analysis to verify if the gastrointestinal lesions were related to the ones in the spleen. RT-qPCR was performed by a technique based on the *TaqMan* probe method, which proved to be positive for *Leishmania spp.* Thus, demonstrating the utility of a high specificity test such as the RT-qPCR (Castelli et al., 2021). With the diagnosis consolidated, Zimba would benefit from RT-qPCR going forward since it can detect and measure parasitic loads before, during and after treatment (Galluzzi et al., 2018).

Serology by ELISA was another important diagnostic method used and turned out positive, demonstrating the presence of Zimba´s *Leishmania spp.* antibodies in its serum. The title was quite high. The kit used was *CIVTEST CANIS LEISHMANIA* which is an indirect ELISA to obtain the 3.4 result (Highly Positive). In this test, after the ELISA procedure is completed, the absorbance of each sample at 450 nm is read. Then, the Rz values of samples are obtained through the kit´s formula and then compared to its cut-offs. Maurelli et al. (2020) suggest the use of recombinant antigens (e.g. KMP11 or LiP) instead of purified *Leishmania spp.* antigens for results with better specificity and sensitivity. Even though IFAT is considered the *gold standard* method for serological diagnosis of canine leishmaniasis, the use of the previously referred antigens in ELISA can lead to more truthful values in a simpler and more practical test than IFAT. (Olías-Molero et al., 2019).

The results obtained through protein electrophoresis, despite Zimba´s not hyperproteinaemic and hyperglobulinemic state, were consistent with the literature (Marques, 2014; Paltrinieri et al., 2016) and demonstrated a polyclonal pattern of the beta and gamma fractions. The alpha-2 being increased can be due to a more acute state of leishmaniasis or another acute inflammatory cause. Even though albumin seems increased in the proteinogram, its total values are below normal levels. A: G ratio was low but still on normal values. It is worth noting that this was his first proteinogram performed and the animal would benefit from more of this analysis to monitor his clinical situation during and after treatment.

No haematological or biochemical indicators were assessed in Zimba´s case. Nevertheless, they should be considered in further analysis to better monitor the clinical course of the disease.

2. IMMUNE-MEDIATED HAEMOLYTIC ANAEMIA

2.1 Anaemia and its classifications

The term ‘anaemia’ refers to a medical condition defined by a decrease of the total erythrocyte mass (either by a decline of red blood cells or a decrease in haemoglobin) resulting in less oxygen reaching tissues. Haematimetric parameters such as red blood cell count (RBCs), haemoglobin concentration (Hb) and haematocrit (Hct) or Packed Cell Volume (PCV) can be measured and will be decreased in anaemic animals (Harvey, 2013). Clinically, anaemia can translate into fatigue, weakness, pale mucosae, cold extremities and irregular heartbeat patterns. Furthermore, depending on the primary cause, other signs may be present, for example, melena in cases of anaemia secondary to gastrointestinal blood loss (Nelson & Couto, 2021).

When diagnosing, classifying and evaluating anaemia, from a clinical pathologist's point of view it's important to notice these measured and calculated values can be altered by certain factors (e.g. dehydration; *in vitro* haemolysis among others), making them appear normal, decreased or increased. It is then mandatory to distinguish a true/absolute anaemia from a relative one. A true anaemia fits the previous description of anaemia, while a relative one can be due to an increase in plasma (e.g. overhydration) (Harvey, 2013). In order to understand anaemia, we have to characterize it by using 3 major pillars: erythrocyte size and haemoglobin concentration; bone marrow response (regenerative/nonregenerative) and pathophysiologic mechanism (Thrall et al., 2012).

When it comes to classifying erythrocyte size and haemoglobin concentration, flow cytometric haematology analysers can provide us with values of mean cell volume (MCV) and mean corpuscular haemoglobin concentration (MCHC). MCV measures erythrocyte volume as it can be normal (normocytic), above reference intervals (macrocytic) or below reference intervals (microcytic). MCHC measures the mean of haemoglobin per unit of volume of red blood cells as it can be normal (normochromic) or below reference intervals (hypochromic). Hyperchromic states (increased haemoglobin) are not possible and are always artefactual. These can generally result from lipemia, intravascular/extravascular haemolysis, agglutination, strong *rouleaux* or Heinz bodies, and it is of utmost importance that clinical pathologists are familiar with these scenarios to avoid erroneous interpretations (Thrall et al., 2012).

The bone marrow´s response is also a key element when classifying anaemia. It allows both pathologists and clinicians to understand if the response is regenerative or nonregenerative by measuring the number of immature RBCs in circulation. A regenerative response usually takes about 2-4 days to be efficient and can happen as a physiological response to factors such as increased erythropoietin production (secondary to hypoxia) or a response to pathological causes such as blood loss or abnormal blood cell destruction (Harvey, 2013). The most useful parameter to assess regeneration is the reticulocyte count. Reticulocytes are non-nucleated immature red blood cells (RBC). These differentiate themselves from other cells when stained with special dyes because they contain at least two blue staining particles or one particle linked to a filamentous thread (Gaur & Sehgal, 2021). The most accurate and fastest way to count reticulocytes is by using flow cytometric haematology analysers with a blood-EDTA sample. Alternatively, an estimate can be achieved by manual reticulocyte count on a blood smear. For this last method, supravital stains such as new methylene blue and brilliant blue cresyl can be used to differentiate and count reticulocytes. Anaemia is considered theoretically regenerative if the reticulocyte concentration is greater than 60 000 cells/µL for both dogs and cats (Thrall et al., 2012). Reticulocyte count, though, needs to be corrected using, for example,

the patient's haematocrit at the time of the reading for a more accurate assessment of the bone marrow's response. This correction, also known as Corrected Reticulocyte Percentage, is essential in order to reduce the dilutional effect of the anaemia on the count. For instance, when given two adult male dog patients, one with a very low haematocrit and the other with only moderate anaemia, the same reticulocyte count may be sufficiently regenerative for the dog with moderate anaemia and insufficient for a dog with a more severe one (Harvey, 2013). The Corrected Reticulocyte Percentage is considered regenerative if a corrected percentage is higher than 1% for dogs or higher than 0.4% for cats (eClinPath, 2013). On a regular stained blood smear, evidence of polychromasia and increased MCV can also support a regenerative bone marrow response (Villiers & Ristic, 2016).

After establishing if the anaemia is regenerative or non-regenerative, it is then possible to consider the different pathophysiological mechanisms leading to it. Non-regenerative anaemias happen when there is a decrease (hypoplasia) or complete cease (aplasia) of erythropoiesis and other cell lineages in the bone marrow. Primary and secondary disorders of the bone marrow such as myelofibrosis and decreased erythropoietin due to chronic renal disease (respectively), are amongst the most common cases. Other known causes include inflammation, infectious agents, immune-mediated destruction of erythrocyte precursors and the effect of drugs/toxins (Thrall et al., 2012). With regenerative anaemia, the most common mechanisms relate to blood loss (e.g. trauma) or erythrocyte destruction (e.g. immune-mediated haemolytic anaemia). Causes of inflammation, infectious agents and drugs are also valid (Thrall et al., 2012) though, as mentioned above, these typically lead to a non-regenerative response.

For the purpose of this chapter, we shall be focusing on anaemia from immune-mediated origin (immune-mediated haemolytic anaemia).

2.2 Immune-mediated haemolytic anaemia

Immune-mediated haemolytic anaemia (IMHA) is a process where erythrocytes are destroyed by an antibody-mediated response (caused either by macrophages or by the formation of a complement membrane attack complex (MAC)). This fact often results in red blood cell agglutination (due to antibody fixation) which may greatly affect the haematimetric parameters obtained by automated analysers (discussed further ahead in this document). Understanding the pathways on which erythrocytes are being destroyed is a crucial point to better classify patients (Thrall et al., 2012). The destruction of RBCs can be extravascular, which is the most common form in dogs, or intravascular (Villiers & Ristic, 2016). Extravascular

haemolysis occurs when macrophages phagocytise RBC by opsonisation due to the IgG (most commonly) or IgM on their surface mainly in the spleen, liver and bone marrow. The entire cell can be fully or partially phagocytized leading to the formation of spherocytes, which are a hallmark of this type of haemolysis. These well-defined, non-pale, spherical-shaped RBCs can only be correctly evaluated in dog blood smears since, in the cat, erythrocytes already do not show a physiological central pallor and are smaller in size making it hard to distinguish normal from abnormal cells (Harvey, 2013).

On the other hand, intravascular haemolysis occurs inside blood vessels mediated mainly by IgM and MAC. This complex formation leads to the damage of the erythrocyte membrane resulting in extracellular fluid influx to the cell and its swelling and destruction. In blood smears, this can be observed in the form of "ghost cells". These two findings are of major importance in the clinical pathology of anaemia cases (Villiers & Ristic, 2016).

Normally, IMHA is markedly regenerative but nonregenerative forms can occur. Examples of this include animals with other comorbidities (for example, a patient with chronic kidney failure, in which even in the presence of immune-mediated anaemia, insufficient erythropoietin is produced in the kidneys) or, a rare cause when the immune-mediated response also affects the most immature precursors inside the bone marrow (Precursor-Targeted Immune-Mediated Anaemia) with the formation of antibodies against erythroid precursors secondary *vs* primary/idiopathic or secondary (Thrall et al., 2012).

To distinguish a primary from a secondary immune-mediated anaemia, a thorough analysis is needed to understand if a known cause can be identified (secondary) or not (primary). The IMHA is the most common cause of haemolytic anaemia in dogs and about two-thirds of dogs diagnosed with it exhibit its primary form (Harvey, 2013). As an example of the secondary form, cases of canine babesiosis have been identified as triggering immune-mediated anaemia, even when the cause is resolved (Garden et al., 2019).

In feline patients, this form of anaemia is more commonly secondary and usually linked to infections by *Mycoplasma haemofelis,* feline leukaemia virus (FeLV), lymphoproliferative and myeloproliferative disorders (Thrall et al., 2012; Garden et al., 2019).

The formation of alloantibodies is also an interesting factor when it comes to immune-mediated haemolytic anaemia. These are antibodies against antigens from other individuals of the same species and tend to occur, most commonly in small animals, during non-compatible blood transfusions. A similar process can also happen in neonatal isoerythrolysis leading to severe cases of anaemia, which is more common in horses (Balch & Mackin, 2007).

Additionally, a temporal association between vaccination and the development of IMHA has been described. Some reports described this condition affecting animals after 30 days of the

vaccination date, suggesting it might be a trigger factor (Balch & Mackin, 2007). However, strong evidence is still lacking and vaccination practices are generally safe and advised in veterinary medicine (Garden et al., 2019).

2.3 Clinical signs

Animals affected by immune-mediated haemolytic anaemia show some signs present in all forms of anaemia such as depression, exercise intolerance, systolic heart murmur, pale mucous membranes and others, with some differences. A typical finding in these patients is the presence of jaundice due to the intense degree of haemolysis (though jaundice can be absent in some cases) (Nelson & Couto, 2021). Acute cases can result in collapse and more severe symptoms, while in chronic ones, animals tend to adapt to the anaemia and show minimal signs of disease (Thrall et al., 2012).

2.4 Laboratory findings

2.4.1 Haematology

Haematological parameters and the findings on blood smears are essential for the diagnostic process of IMHA. Expected changes in haematimetric values are the decrease in both red blood cell count (RBC) and haematocrit (Hct) or packed cell volume (PCV) due to the intense haemolytic process (Thrall et al., 2012). Another interesting finding in these patients is the mean cell haemoglobin concentration (MCHC), which measures the total haemoglobin concentration per unit of volume of the existing RBC population. This value is usually artifactually increased and there are two principal situations that contribute to this false result. The first is, due to the agglutination process, the automated machine fails to correctly count many of the erythrocytes, reading less than reality, reporting a very low RBC count, while counting all the available haemoglobin, seemingly reporting more haemoglobin to very little red blood cells. Another process is, due to the haemolytic nature of this condition, especially when intravascular, there's destruction of RBCs resulting in more free haemoglobin to the proportion of circulating RBCs. This proportion translates into a higher MCHC than physiologically possible (eClinPath, 2013).

Prominent spherocytosis, as mentioned above, is not only an important finding but also a hallmark of extravascular haemolysis and of major importance in the diagnosis of IMHA. These spherical, well-defined, RBCs can only be correctly evaluated in dog blood smears since, in the cat, erythrocytes are smaller and have no central pallor making it hard to distinguish normal from abnormal cells. According to MacNeill et al. (2019), observing 5 or more spherocytes per 100x oil immersion field supports the diagnosis of IMHA. However, spherocytes can also happen

physiologically in cases of post-transfusion or even in cases of increased oxidative damage, envenomation, pyruvate kinase deficiency and hypersplenism. Thus, knowing the clinical history of patients becomes a crucial point in the correct interpretation of this finding, along with other haematological patterns (such as agglutination) (Garden et al., 2019).

In addition to the prominent spherocytosis, in the blood smear, marked anisocytosis and polychromasia are common due to the different sizes of spherocytes and erythroid precursors and the haemoglobin concentration of RBCs in regenerative anaemia cases. (Villiers & Ristic, 2016). If agglutination is spotted, it can be confirmed by a positive saline test, as discussed below.

2.4.2 Positive saline agglutination test

Agglutination is a common finding in IMHA due to the intense antibody-antigen connections that are established. True agglutination needs to be distinguished from intense *rouleaux*. The most used, cheap, and practical method for this is the Positive Saline Agglutination test (SAT) which helps with this distinction. *Rouleaux* can form in situations of inflammation where a high globulin content is produced (though healthy cats and horses may also physiologically have a few increased *rouleaux*). Microscopically it usually appears as a stack of coins in the smear, though it's not easy to always distinguish from agglutination just by blood smear evaluation (Sun & Jeffery, 2020).

In order to perform a saline test, a drop of blood is mixed with a saline solution. The test is considered negative if the RBC's agglutinates dissipate, confirming *rouleaux*. If, on the other hand, erythrocytes stay bounded, even after increasingly higher dilutions, true agglutination can be assumed. In most cases, a dilution of 4 drops of saline solution (NaCl 0.9%) to 1 drop of non-coagulated blood is performed at around 37ºC to obtain a valid result. To confirm true agglutination in the following situations, washing erythrocytes 3 times with a 4:1 saline-to-blood ratio should be considered: equivocal results, markedly increased total protein or fibrinogen and strong *rouleaux* formation. (Garden et al., 2019).

According to Sun & Jeffery (2020), SAT achieves higher specificity for the diagnosis of IMHA when a 49:1 saline-to-blood ratio is used at a temperature of 37ºC. With the SAT, it is mandatory to firstly rule out all of the other possible causes of agglutination for a right interpretation of auto-agglutination typical of IMHA.

2.4.3 Antiglobulin Direct tests

Actual evaluation of the presence of antibodies on the membranes of erythrocytes can only be done in laboratories by another test: the direct Coombs test. It's part of the group of Antiglobulin Direct Tests (DAT) and detects the presence of immunoglobulins and/or complement bound to RBCs on blood samples (Warman et al., 2008). Different forms include a microtiter plate format, gel-based microcolumn or capillary DAT, all of which are valid. The most used is the microtiter plate format, resulting in auto-agglutination as a positive result when mixing a blood sample and the Coombs reagent (species-specific) in increasingly higher dilutions. A positive result comes in the form of a clump of suspended cells that need to be microscopically verified to confirm true agglutination. Serial dilutions are key to mitigate the possible prozone effect, in which there is an excessive amount of antiglobulin compared to the antibodies present in the RBCs membrane, leading to non-agglutination and thus a false negative (Villiers & Ristic, 2016). Because antibody binding is temperature-dependent, it is recommended that this procedure should be performed at both 37ºC and 4ºC. Important to note that, even though Coombs was considered an almost mandatory test for IMHA diagnosis, according to the latest ACVM Consensus (2019), DAT may not be performed if all of the following are verified: agglutination persists after washing, anaemia and evidence of haemolysis. (Garden et al., 2019). Furthermore, knowing the clinical history of the patient is crucial because recent blood transfusions and immunosuppressive treatment can induce false positive and false negative results, respectively. (MacNeill et al., 2019)

In DAT, it is mandatory to first rule out all of the other possible causes of agglutination for a right interpretation of the typical auto-agglutination related to IMHA.

2.4.4 Leukogram

Animals with IMHA usually present a typical inflammatory pattern justified by the pro-inflammatory state that this condition provides. Findings as neutrophilia with a left shift (with or without toxicity) lymphopenia, eosinopenia and monocytosis can occur. In more severe cases a leukoerythroblastic pattern can be observed with highly acute inflammatory leukogram and an increased amount of nucleated RBCs (MacNeill et al., 2019).

2.4.5 Platelets and coagulation

Thrombocytopenia is a common finding in IMHA patients, reported in up to 65% of dogs (Villiers & Ristic, 2016). Since most dogs with IMHA are in a hypercoagulable state, it becomes mandatory to determine what is causing the increased consumption of platelets in these

animals. The most common differentials are disseminated intravascular coagulation (DIC) and pulmonary thromboembolism (PTE). These findings can be of major importance because they end up being one of the main causes of death in animals who suffer from IMHA. Causes to consider also include immune-mediated thrombocytopenia (IMT) and some cases may present IMHA and IMT in a condition known as Evan´s Syndrome (MacNeill et al., 2019).

Coagulation parameters often encountered are prolonged prothrombin time (PT), prolonged activated partial thromboplastin time (APTT), decreased fibrinogen and increased concentration of D-dimers (Thrall et al., 2012).

2.4.6 Biochemistry and urinalysis

Biochemical parameters can vary with the nature of the anaemia process and several studies report different findings.

A common finding in IMHA patients is increased total bilirubin (hyperbilirubinemia) (MacNeill et al., 2019). This is explained by the intense haemolysis these animals are subjected to, as well as this haemolysis being mostly extravascular (Villiers & Ristic, 2016). It is important to be alert for clinical signs like jaundice, as well as rule out other possible causes of increased total bilirubin: decreased functional hepatic mass, obstructive cholestasis and sepsis (Garden et al., 2019).

Still related to the intense haemolytic nature of this condition, hemoglobinemia and haemoglobinuria are also possible findings (MacNeill et al., 2019). These can be more suggestive of intravascular haemolysis. Factors that affect erythrocyte fragility (e.g. lipemia) can mislead the operator because they can cause *in vitro* haemolysis making it important to establish where RBC destruction is occurring (Villiers & Ristic, 2016).

Another possible discovery is renal damage (MacNeill et al., 2019). This occurs through mechanisms such as hypoxia, the deposition of antibody-antigen complexes on renal tissue or nephrotoxicity of free haemoglobin (Thrall et al., 2012). Azotaemia and proteinuria can also be observed (Moraes et al, 2017).

2.4.7 Flow cytometry

Flow cytometry has proven to be a really useful tool when diagnosing IMHA as it can detect immunoglobulins bound to erythrocytes (MacNeill et al., 2019).

This diagnostic method is reported to be, not only more sensitive than the direct Coombs test but also easier and quicker to perform (Moraes et al, 2017). Because of the

quantitative nature of the result obtained, it can be used to better monitor patients (Garden et al., 2019).

2.5 Diagnosis

Immune-mediated haemolytic anaemia doesn't have a diagnostic gold standard test. Instead, several parameters are used to reach this diagnosis and are schematically described by Garden et al. (2019) and represented in Figure 1.

In a summarized approach, the first step is to determine if the animal is anaemic. Then it's mandatory to identify signs of immune-mediated destruction and haemolysis. Important to note that the other anaemia causes must be ruled out. Genetics can also play a part in the diagnostic process, especially in dogs where certain breeds are described as more predisposed: Cocker Spaniel, English Springer Spaniel, Miniature Schnauzer and Old English Sheepdog. (MacNeill et al., 2019)

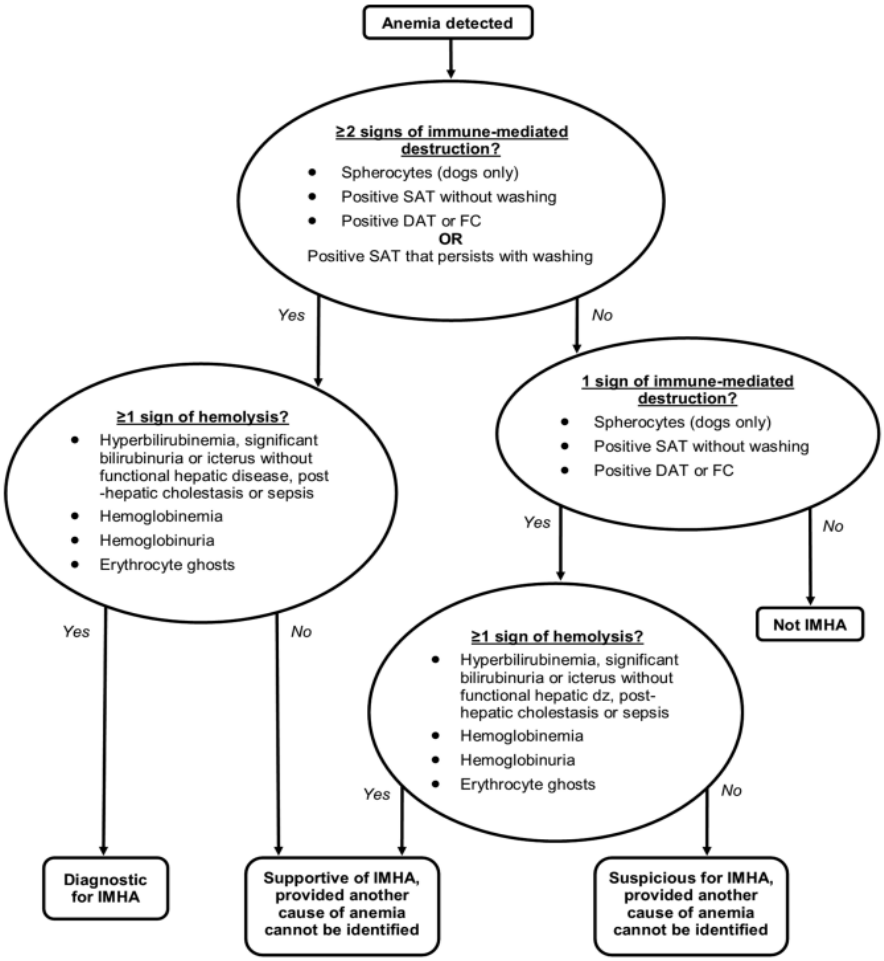


Figure 9 - IMHA Diagnosis Algorithm. Adapted from Garden et al. (2019)

2.6 Clinical case: Duda (reference intervals present in attachments)

Patient: Duda is a 15-year-old mixed-breed female with a suspicion of anaemia.

Exams performed: Hemogram, reticulocyte count, blood smear evaluation**,** direct Coombs test and saline agglutination test.

Results:

- Reported Hematocrit (automated machine): 3.7%(↓); Haemoglobin: 3.4 g/dL
- Manual microhematocrit: 10% (↓), slightly icteric plasma, non-lipemic, presence of macro-agglutination.
- MCV: 92.5 fL; MCHC: 91.9 g/dL
- Blood smear evaluation
    - Erythrogram: Marked anisocytosis, polychromasia, spherocytosis and suspected micro-agglutination. Several immature nucleated RBCs and rare ghost cells. It is represented in Figures 2 and 3.
    - Leukogram: Normal leukocyte density (15.24x10^9/L). However, band neutrophils (10.55 x10^9/L) are observed with mild to moderate signs of toxicity. Mild monocytosis (3.30x10^9/L; ↑) with some activated monocytes.
    - Plateletgram: Adequate density (268x10^9/L) and morphology.
    - No parasites or abnormal cells were observed.
- Saline Agglutination test - Positive (4:1)
- Direct Canine Coombs test - Positive
- Reticulocyte Count - 101.0x10^3/µL (↑)
- Corrected reticulocyte percentage: 5.59%

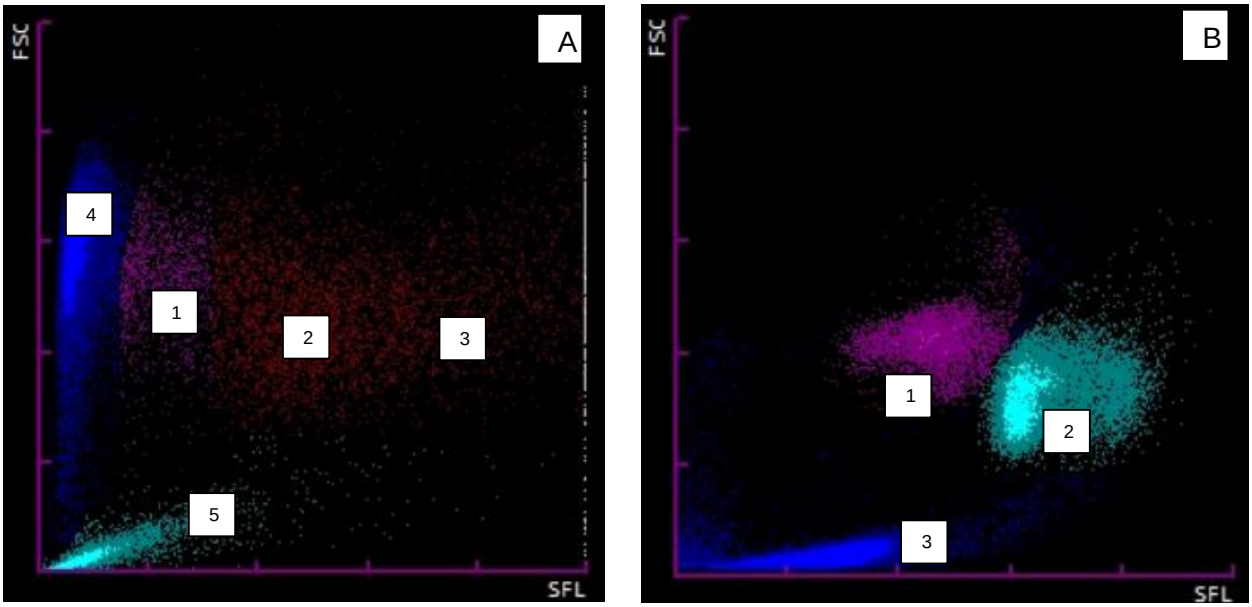


Figure 10 - Scattergrams of Duda´s blood sample. A) Reticulocyte maturity fractions in the reticulocyte channel scattergram showing the different populations of nucleated RBCs. (1) LFR (low-fluorescence reticulocytes) or 'mature' reticulocytes, (2) MFR (medium-fluorescence reticulocytes) or 'semi-mature' reticulocytes, (3) HFR (high-fluorescence reticulocytes) or 'immature' reticulocytes, (4) Non-nucleated RBCs and (5) Ghost population.; B) Nucleated populations on the WNR channel scattergram. (1) Nucleated erythroid population, (2) leukocyte population and (3) Ghost population. Axis: Y represents Forward-scatter light (FSC) - cell volume and X represents Side-fluorescence light (SFL) - the amount of nucleic acids and cell organelles. Obtained from Sysmex XN-V haematology analyser and based on (Brown et al., 2023; Ginders et al., 2024).

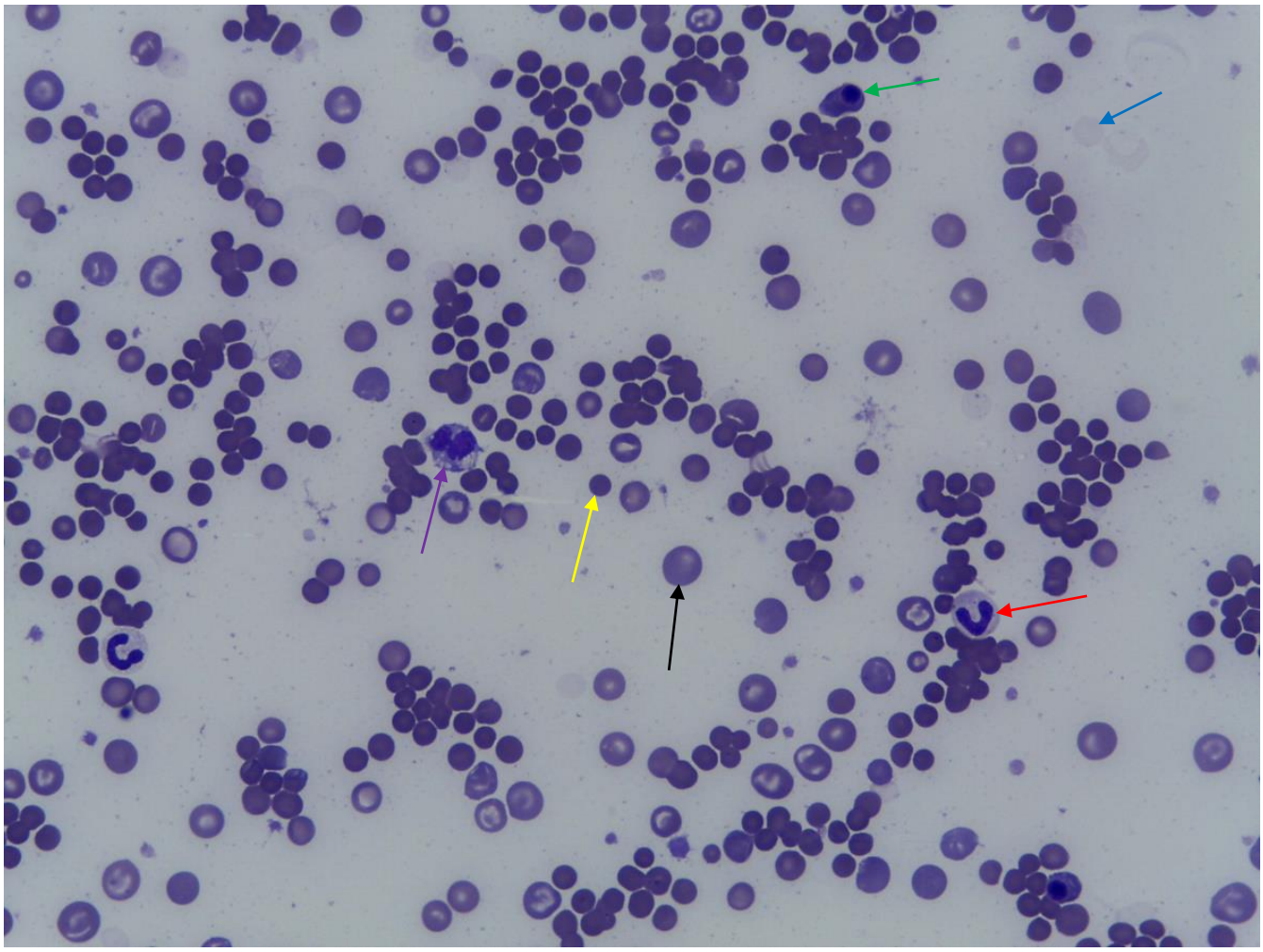


Figure 11 - Duda´s blood smear. Marked polychromasia and anisocytosis with prominent spherocytosis (yellow arrow). Two metarubricytes (green arrow) are observed alongside several polychromatophilic erythrocytes (black arrow). Band neutrophils with basophilic cytoplasm and Döhle bodies (red arrow) are present with also activated monocytes with clear vacuoles (purple arrow). Note the few existing erythrocyte ghost cells on the background (blue arrow). May-Grunwald Giemsa (MGG). 40x

Conclusion and Observations: The results obtained were compatible with immune-mediated haemolytic anaemia, supported by a positive Coombs. Further exams and follow-ups of Duda´s condition should be made to better understand the cause of her anaemia: primary (auto-immune), alo-immune (e.g. secondary to blood transfusion) or secondary (e.g. secondary to infectious agents or neoplasia). Leukogram revealed a left shift and signs of toxicity with no neutrophilia that can indicate the existence of an inflammatory response where the production and consumption of neutrophils are balanced. Monocytosis can occur in cases of inflammation (chronic or acute), under corticosteroid influence and still in cases of IMHA.

2.7 Discussion and the role of Clinical Pathology

This discussion will mainly be based on Garden´s algorithm for the diagnosis of IMHA in a well-collected blood-EDTA sample.

For starters, the blood-EDTA tube presented macro-agglutination, clearly visible to the naked eye. This finding has already been reported in cases of IMHA and is of major importance when interpreting the haematologic analysis that will follow. Upon this scenario, it is always very important to verify if there is also micro-agglutination and distinguish it from being true agglutination or intense *rouleaux*. For this, the saline agglutination test was then performed and will be later discussed (Sun & Jeffery, 2020). After obtaining the hemogram, several of the already discussed above topics were demonstrated. Duda´s haematocrit, as reported by the automated analyser was 3.7% which is very low and compatible with severe anaemia since normal values for dogs go between 37.0 – 55.0%. Because this haematocrit is not compatible with life and macro-agglutination was observed in the tube, this number was most definitely not real. The microhematocrit revealed the real haematocrit value to be 10%, which is still anaemia, but not so severe. Haemoglobin (3.4 g/dL) was another factor that was decreased since normal values go from 12.0 – 18.0 g/dL (Thrall et al., 2012). The MCHC was 91.9 g/dL when average values go from 31.0 - 36.0 g/dL in dogs. Artefactual increase of the MCHC can be suggestive of lipemia, intravascular/extravascular haemolysis, Heinz bodies, or agglutination (Thrall et al., 2012). For this last reason, RBC agglutination results in clumps of erythrocytes which are not properly read by impedance automatic counters, leading to an increase in the proportion between the amount of free haemoglobin and the total erythrocyte mass. In other words, fewer erythrocytes are counted for the same amount of haemoglobin (MacNeill et al., 2019). At this point, because the smear had not been evaluated yet, only lipemia could be ruled out, since it can easily be seen macroscopically. Sometimes, we can evaluate it after performing the microhematocrit and appreciating a fat layer (eClinPath, 2013). Upon viewing the blood smear, the presence of spherocytes and ghost cells helped to justify the increased MCHC and the existence of real haemolysis. The presence of micro-agglutination was also verified as another factor for the increased MCHC (Castilho et al., 2016).

The MCV reported in this case was 92.5 fL which is above average for dogs with normal values being from 60.0 – 77.0 fL. This can be easily explained by the presence of erythroid precursors counted by the impedance automatic counter because these are usually bigger cells than the already mature erythrocytes (Harvey, 2013).

Flow cytometric evaluation of Duda´s blood revealed, in both graphics, an existing population of nucleated RBCs with a strong regenerative response incorporating all the different fractions of reticulocytes (Mendoza & Dvm, 2021). A high number of nucleated erythrocytes in

peripheral blood is usually seen in regenerative responses of the bone marrow typical of situations such as immune-mediated haemolytic anaemia, secondary to blood loss or even cases of toxicity, splenic disorders, and neoplasia, among others. In Duda´s case, it is mostly likely due to a regenerative response secondary to her haemolytic anaemia of immune-mediated origin. (Brown et al., 2023; Ginders et al., 2024).

The absolute reticulocyte count of 101 000 cells/µL demonstrated a regenerative response, which is considered when this count is greater than 60 000 cells/µL in dogs (Thrall et al., 2012). Due to the already observed agglutination, the corrected reticulocyte percentage was calculated to determine if the anaemia was truly regenerative using the PCV from the microhaematocrit (Balch & Mackin, 2007). The result was 5.59%, proving, in fact, a regenerative response from the bone marrow to the anaemia since the result is bigger than 1% (eClinPath, 2013).

Under the microscope, the blood smear examination revealed marked anisocytosis, polychromasia and agglutination. Prominent spherocytosis was present with the criteria of 5 or more spherocytes per x100 oil-immersion field being matched. Ghost cells were also present, despite not being a lot (MacNeill et al., 2019). While it could be a sign of intravascular haemolysis, it is most likely just an artefact from lysed RBC related to smear preparation (Harvey, 2013). Both findings prove the occurrence of haemolysis, mostly extravascular due to the larger amount of spherocytes. Still, in the smear examination, the non-presence of parasites can suggest a more primary form of IMHA, but more tests should be performed (e.g. PCR) and all the other causes of secondary IMHA should be ruled out (Garden et al., 2019).

In addition, SAT and Direct Coombs tests were performed and both were positive, demonstrating the existence of true auto-agglutination in the sample. SAT was done in a 4:1 saline-to-blood ratio and DAT was performed on its capillary form. Further, SAT could have been made in increasingly higher dilutions to assure a more truthful result (more specificity) (Sun & Jeffery, 2020). Sadly, there was no measurement of proteins (albumin e globulins) in Duda´s case since it would be a piece of additional information, alongside SAT, to better distinguish true agglutination from *rouleaux*, since this last one forms in increased globulin situations (MacNeill et al., 2019).

Another, not so important, finding in Dudas's case is related to her leukogram which fits the ones described in IMHA literature: band neutrophils with mild to moderate signs of toxicity (left shift with toxicity) and mild monocytosis with some activated monocytes (MacNeill et al., 2019).

Finally, it would be interesting to assess the existence of haemolysis. In this case to measure, at least, the total bilirubin concentration in the blood. This is due to the sample being

slightly icteric and icterus/jaundice can be perceived clinically when total bilirubin exceeds 3 mg/dL. The measurement of conjugated/direct bilirubin would also add information to this situation since it is likely that the hepatic threshold for turning unconjugated bilirubin into its conjugated form has been surpassed and we are already seeing signs in the plasma. Another possibility to better understand this possible hepatic overload is urinalysis and the presence of bilirubin in urine. Careful must be taken, since dogs excrete a small and physiological amount of it normally. Aside from these two, icterus without functional hepatic disease, post-hepatic cholestasis or sepsis can also be perceived as a sign of haemolysis. To better understand these parameters, bile acids and urea (for functional hepatic disease) and even diagnostic imagining (for post-hepatic cholestasis) can be measured/performed (EClinPath, 2013; Stockham & Scott, 2013).

Duda gathers strong evidence (more than two signs) of immune-mediated destruction, but still no clear sign of real haemolysis because the ghost cells present can be artefactual as stated before. Therefore, the measurement of parameters such as total bilirubin concentration becomes mandatory to reach a final diagnosis of IMHA according to the clinical pathology standards. Sadly, no clinical information about her was given, but the presence of clinical signs such as depression, exercise intolerance, heart murmur, pale mucous membranes, and other signs related to anaemia could help establish the diagnosis of IMHA (Garden et al., 2019).

3. FELINE LYMPHOMA

3.1 Introduction and aetiology

Lymphoma is a broad term since it incorporates a wide variety of malignant proliferation of lymphoid cells arising from lymphoid tissue outside of the bone marrow with a multifactorial aetiology (Zachary & McGavin, 2011). It is the most frequent haematopoietic cancer in cats representing an annual incidence of up to 48 cases per 100.000 animals (Economu et al., 2021). Factors that contribute to its appearance in this species range from breed, the environment patients are exposed to, and their FeLV or FIV infection status, with studies reporting a decreased chance for lymphoma development in FeLV-vaccinated cats. There is a sex-apparent predisposition for males (according to some studies), and it is a predominantly present cancer in middle-aged to older cats (Economu et al., 2021).

In small animal medicine, several methods are used for the diagnosis of lymphoma. In recent years, cytology has been found to play a major role in disease screening. According to the Kiel classification system, this quick, relatively cheap and sensitive technique can be useful for diagnosing high-grade lymphomas. However, in most cases, further characterization is needed

to choose the most adequate treatment plan, hence tissues need to undergo histopathological examination (Gambini et al., 2021). Histopathology allows for a classification according to the World Health Organization system, subdividing lymphomas into several groups based on cell features such as their origin, stage of maturation, size, and histologic pattern (Meuten, 2016). Another form of lymphoma classification is through flow cytometry or even with PCR assay for antigen receptor rearrangement (PARR), with the first being a superior alternative. These classifications help clinicians to better monitor and give prognostic to their patients (Economu et al., 2021).

Regarding lymphoma classification, two interesting points to clarify are cell size and type. These neoplasms are characterized by size as small, medium and large and the size reference of the average well-differentiated small lymphocyte is the normal erythrocyte. More immature lymphoid cells are classified as medium and large, as they are 1.5 to 2 times or 2 to 3 times bigger than the normal erythrocyte. Interestingly, cats tend to have more small-sized lymphomas while dogs tend to have more large-sized ones (Valenciano & Cowell, 2020). Another major part of lymphoma´s classification in order for better treatment and prognosis comes from determining its immunophenotype: B-cell, T-cell, both cell lineages or neither. In cats, the prognostic value of immunophenotyping is scarce and the most useful information tends to be: the patient´s retroviral status, anatomic location, and initial response to therapy (Twomey, 2005). However, this step is especially important in the case of canine lymphoma, since T-cell lymphomas have a shorter remission and survival time than B-cell lymphomas (Zandvliet, 2016). Other aspects regarding classification will be explored later in this chapter.

When it comes to feline medicine, it’s important to take note that feline lymphoma is recognisably more challenging to diagnose than its canine counterpart. This is due to factors such as its location being more common in internal organs and not in peripheral lymph nodes and in some cases, the population of lymphocytes being more heterogeneous than the classical descriptions of these neoplasms for canine patients (Burkhard & Bienzle, 2013).

For the purpose of this chapter and the chosen case, feline lymphoma will be the main focus.

3.2 Clinical presentation

The classification of lymphomas can be achieved or partially achieved according to the clinical presentation. This can be highly variable and usually clinical signs will reflect the anatomic location of the neoplastic lesion. In cats, known classifications based on location include alimentary/gastrointestinal lymphoma, mediastinal, multicentric, and extranodal

(Nelson & Couto, 2021). These forms can later be staged regarding other affected organs, blood and bone marrow dissemination (Vail et al., 2019).

The most common form of lymphoma in domestic cats is the alimentary/gastrointestinal lymphoma, diagnosed in up to 55% of cases. This type of disease can be confined or present as a combination of intestinal, mesenteric lymph node, and hepatosplenic involvement. Anatomically it's more frequently found in the small intestine and can be sub-classified into three major categories: low-grade alimentary lymphoma (LGAL), intermediate or high-grade alimentary lymphoma (I/HGAL), and large granular lymphoma (LGL). From these three, LGAL accounts for 80% of cases and it´s majorly of T-cell origin. All alimentary lymphomas can exhibit nonspecific gastrointestinal signs but only LGAL has an indolent clinical progression. It is also worth mentioning that LGL has the worst prognosis with a median survival time of 45-90 days after diagnosis (Vail et al., 2019). Figure 1 better summarizes the characteristics of feline alimentary lymphoma.

Extranodal lymphoma is the second most common form in cats, usually developing in the nasal region or kidney (renal). Clinical signs of this type will correlate to the anatomical site, but may also be nonspecific. An example can be the renal lymphoma where signs of renal failure are predominant (Vail et al., 2019).

Mediastinal lymphoma may cause clinical signs relating to the compressive nature of the enlarged mass/lymph nodes on the surrounding thoracic structures, resulting in dyspnoea, coughing, dysphagia or regurgitation. Edema of the neck and head and pleural effusions are also reported. (Nelson & Couto, 2021). This subtype of lymphoma is usually from a T-cell origin (Vail et al., 2019).

Cutaneous lymphoma can be as epitheliotropic or non-epitheliotropic and it is generally rare in felines. This classification is done histologically (and not exclusive to this subtype), and these are usually also from a T-cell origin (Nelson & Couto, 2021).

Contrary to dogs (in which the multicentric form of lymphoma accounts for about 80% of cases), in cats the multicentric form represents just 10% of lymphoma cases with peripheral lymphadenopathy being mostly uncommon (Vail et al., 2019). Usually in cats peripheral lymph node enlargement is due to the non-neoplastic atypical follicular lymphoid hyperplasia. This syndrome courses often with fever and polyclonal gammapathy (Twomey, 2005).

It is noteworthy that these neoplastic disorders may mimic many others and the origin of the lymphoproliferative malignant process should be assessed in order to distinguish it from, for example, leukaemia (Nelson & Couto, 2021).

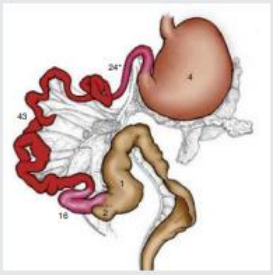
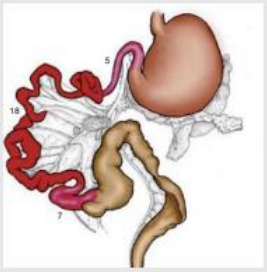
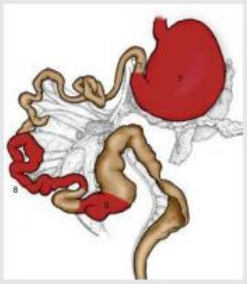
Characteristic	Low-Grade Alimentary Lymphoma (LGAL)	Intermediate-/High-Grade Alimentary Lymphoma (I/HGAL)	Large Granular Lymphoma (LGL)
Incidence	50%–80% of cases	≈20% of cases	≈10% of cases
Clinical presentation	Nonspecific gastrointestinal signs (anorexia, weight loss, diarrhea, inappetence)	Nonspecific gastrointestinal signs; vomiting common if gastric; hematochezia more common if large bowel	Nonspecific gastrointestinal signs; vomiting more common
Clinical course	Indolent clinical progression	Acute clinical progression	Acute clinical progression
Abdominal palpation	Generally normal, modest intestinal thickening and abdominal lymphadenopathy possible	More common to palpate gastric/intestinal mass, mesenteric lymphadenopathy, organomegaly	More common to palpate gastric/intestinal mass, mesenteric lymphadenopathy
Abdominal ultrasound findings	Often unremarkable; diffuse intestinal wall thickening if present is limited to muscularis propria /submucosa; normal intestinal wall layering; mild lymphadenopathy/organomegaly possible	More commonly thickened transmural intestinal wall; loss of normal intestinal wall layering; mass effect more likely; mesenteric lymphadenopathy more likely	More commonly thickened transmural intestinal wall; loss of normal intestinal wall layering; mass effect more likely; mesenteric lymphadenopathy more likely; effusion uncommon but more likely
Topography[a]			
General diagnostics	Cytology generally not helpful; biopsy (full thickness preferred, but endoscopic helpful) with histopathology, immunophenotype, and clonality analysis often helpful to differentiate from LPE	Cytology (mass/lymph node) often diagnostic; biopsy with histopathology, immunophenotype, and clonality analysis less commonly required.	Cytology (mass/lymph node) often diagnostic; biopsy with histopathology, immunophenotype, and clonality analysis less commonly required.
Cell size	>80% small, <20% large	>90% intermediate/large	Intermediate/large
Immunophenotype	>80% T-cell ($CD3^+$)	≈100% B-cell ($CD79a^+$)	Cytotoxic T-cell ($CD3^+/CD8^+/CD79a^-$), or NK cell ($CD3^-/CD79a^-$); often $CD103^+$ and granzyme B^+
Clonality	>90% clonal or oligoclonal	>70% clonal or oligoclonal	>90% clonal or oligoclonal
WHO EATCL classification	90% type II (mucosal) 10% type I (transmural)	90% type I (transmural) 10% type II (mucosal)	≥90% type I (transmural)
Epitheliotropism	Common	Rare	Common
Recommended treatment	Chlorambucil/prednisolone	CHOP- or COP-based chemotherapy; surgery considered if large discreet lesion prechemo; surgery performed if obstruction/perforation	CHOP- or COP-based chemotherapy; surgery considered if large discreet lesion prechemo; surgery performed if obstruction/perforation
Chemotherapy response and outcome	>80% response; median survival 1.5–3 years	≈50%–60% response (30% CR); median survival 3–10 months; more durable if CR	≈30% response; median survival 45–90 days; occasionally more durable

Figure 12 - Feline gastrointestinal lymphoma. Adapted from Vail et al., 2019

3.3 Regarding FeLV infection

Feline Leukemia Virus (FeLV) is one of the most important conditions in feline medicine and it is also strongly associated with the development of lymphoma (Silva et al., 2022). Since Portugal has one the highest prevalence of FeLV among EU countries (8.8%), it is a point of veterinary concern when talking about feline lymphoma (Studer et al., 2019). This RNA virus is highly pathogenic and responsible for mortality in domestic cats through its tropism for lymphoid tissue and immunosuppressive capabilities. By inhibiting T and B cell function, altering monocyte morphology and distribution, and impairing cytokine production and responsiveness, FeLV predisposes to opportunistic infections and neoplasia. Thus, FeLV-infected cats are 60 times more likely to develop lymphoma than non-infected ones, with the most common forms associated with the virus being: mediastinal, multicentric, spinal, renal, and ocular. These are majorly of T-cell origin and usually, cats are under 4 years of age (Sykes & Hartmann, 2020).

When attempting to diagnose a FeLV infection, the clinician must be aware that there are three forms of the disease: progressive, regressive and abortive. The progressive form, having the worst prognosis ends up being the only one that is usually antigen-positive with no antibodies present. Regressively infected cats become harder to detect since viral RNA can be reverse-transcribed into proviral DNA and then be incorporated into the host´s genome after an immune response that suppresses viral replication. In these cases, cats will test negative for FeLV antigen and positive for the proviral DNA and the presence of antibodies. These animals later in life, can develop a progressive and more aggressive form of the disease with bone marrow involvement. Therefore, they are more predisposed to lymphoma and leukaemia in later life stages. Abortive cases are less common, with cats having antibodies but neither the antigen nor proviral DNA present (Charalambous et al., 2024). It's always advisable to do both antigen and proviral DNA testing for a more complete panel (Sykes & Hartmann, 2020).

Vaccination and testing have had a big impact on this disease over the last decades, allowing FeLV cases to greatly decrease. When relating it to feline lymphoma this case decrease becomes evident since, while in the 1980s, around 70% of cats with lymphoma were associated with FeLV, nowadays, more than 80% of lymphoma patients test negative for the retroviral antigen (Sykes & Hartmann, 2020).

3.4 Diagnosing feline lymphoma

3.4.1 Haematology and biochemistry

The clinical pathological findings in feline lymphoma patients are mostly nonspecific and secondary to the disease. Mild to moderate anaemia can be observed in several forms of lymphoma or secondary to another condition that can coexist with it. Neutrophilia, hypoproteinemia, hypoalbuminemia and hyperglobulinemia, among others, are common findings in GI lymphoma (Vail et al., 2019).

Additionally, because of hepatic involvement in some cases, liver enzymes such as Alkaline Phosphatase (ALP) can be increased. Azotaemia was reported to be typical and more significant in renal lymphomas (Gabor et al., 2006).

3.4.2 Cytology and immunocytochemistry

Cytological examination of lymph nodes, masses/organs and effusions has proven to be a quick, sensitive and minimally invasive technique to diagnose lymphoma. Lymphoma is typically characterized by a homogenous clonal expansion of lymphoid cells that replace the normal organ (lymph node or not) population. Cytologically lymphomas can be classified in low

or high grade according to their cell size and mitotic index (Villiers & Ristic, 2016). For a cytological diagnosis to be achieved, it is of uttermost importance that samples are adequately cellular, well-preserved and that inflammation isn't present (Valenciano & Cowell, 2020).

A distinctive feature of feline lymphomas is that, in this species, small to intermediate-sized cell neoplasia is much more common and lymphoma is usually not confined to the lymph nodes, being present in organs of the GI tract, kidneys, nasal cavity and others. These factors make the diagnosis more difficult not only because they may be similar to inflammatory infiltrates of mature lymphocytes but also because these samples tend to be more heterogeneous than the average lymphoma. Thus, cytological evaluation, compared to dogs where most lymphomas arise from the lymph nodes and have a more classical pattern, can be challenging (Burkhard & Bienzle, 2013).

In high-grade lymphomas, neoplastic cells tend to be larger than average lymphocytes and neutrophils, have finely granular dispersed chromatin, one or multiple small to medium-sized and prominent nucleoli, a lower N: C ratio, and basophilic cytoplasm. Other features may include pleomorphic nuclei, binucleation or clear punctate cytoplasmic vacuoles. When it comes to the mitotic index, a moderate to high mitotic rate of more than 3 mitotic figures per 5 high-power fields is expected. Tingible bodies are usually present within macrophages and numerous lymphoglandular bodies (cytoplasmatic fragments) throughout the sample (Valenciano & Cowell, 2020; Villiers & Ristic, 2016).

Low-grade lymphomas have a predominance of a neoplastic homogenous population of small mature lymphocytes. When it comes to mitotic index, these lymphomas tend to have less the 1 mitotic figure per 5 high-power fields. These are usually more challenging to diagnose since inflammatory conditions such as inflammatory bowel disease also present an infiltrate of small to medium lymphocytes. This challenge can be encountered both in cytology as well as sometimes in histopathology. For these cases, complementary exams such as PARR or flow cytometry are needed to aid the differentiation (Valenciano & Cowell, 2020; Villiers & Ristic, 2016).

Using immunocytochemical markers such as the membranous CD3 for T-cell and the membranous CD79-alpha or nuclear PAX-5 for B-cell, we can reach an immunophenotype and better characterize the pathological process to better administrate treatment to our feline patients (Sampaio et al., 2023).

When it comes to the cytological examination of effusions, lymphoma can sometimes be suspected (but never fully diagnosed) by this method. The presence of neoplastic cells in fluids usually indicates that there is an exfoliating primary mass that needs to be investigated and, only then, properly diagnosed and classified. When a homogenous and relevant population

of lymphocytes as the ones described previously in high-grade lymphoma is present, lymphoma is more easily suspected (especially in feline patients with the mediastinal form and associated neoplastic effusion). However, when small mature lymphocytes are the predominant cell type, other differentials must be taken into account aside from lymphoma, e.g. chylous effusion differentials (Raskin et al., 2022).

3.4.3 Histopathology and immunohistochemistry

Histopathological examination of lymph nodes and other tissues is one of the best methods to achieve an accurate and detailed lymphoma diagnosis. It focuses on several parameters such as cell origin, stage of maturation, size, immunophenotype, and histological pattern. The most popular and consensual classification system most pathologists follow is the World Health Organization system (Zachary & McGavin, 2011).

Gastrointestinal lymphomas are particularly relevant in the cat since it is the most common form of this haematopoietic neoplasm group. Lymphoma can be present in several parts of the GI tract but some studies have the jejunum as a predominant location. Also, it is more often of T than B cell origin since just less than 10% of the villous lamina propria lymphocytes are B (Moore & Kass, 2012). The histological pattern allows pathologists to conclude about lymphocyte infiltration and tropism to epithelial tissue. On the matter of neoplastic cell infiltration, lymphomas can be mucosal (limited to the mucosa and submucosa of the intestinal wall) or transmural (involving the entire thickness and layers of the intestinal wall). This can affect prognosis since transmural lymphomas have a shorter survival time. Tropism to epithelial tissue does not hold much prognostic value in felines and can be classified as epitheliotropic and non-epitheliotropic. Most alimentary feline lymphomas tend to be epitheliotropic from T origin (Meuten, 2016; Moore & Kass, 2012). Other forms of lymphoma can also be diagnosed by histopathology to better describe lesions. (Vail et al., 2019).

Immunohistochemistry, using mostly the same markers for B and T cells as the ones described in cytology, is how immunophenotype can be assessed. Immunohistochemistry is considered the *gold standard* for this matter and a useful tool since it can also apply molecules such as Granzyme-B for the identification of the granules in Large Granular Lymphoma (LGL) cases or even KI-67 to estimate cell proliferation. This last one can be of major importance on the challenging differential between small cell lymphoma and chronic inflammation (Marsilio et al., 2023; Meuten, 2016).

3.4.4 PARR

The PARR is the most used PCR technique in feline lymphoma diagnosis. To better explain this method, in the normal process of maturation of lymphocyte antigen receptors, these acquire high variability through V(D)J rearrangements. The addition and depletion of nucleotides between the V and J segments creates even more variability. This junction, known as the complementary determining region (CDR3) can also be diverse in its composition and codes/determines the lymphocyte specificity, being unique for each cell. PARR ends up targeting this specific zone and, by using forward primers for V genes and reverse primers for J genes, it can infer not the constitution, but the length of the CDR3 portion. In cases of inflammation or reactiveness, lymphocytes tend to come from different cells, having different CDR3 lengths, resembling polyclonal patterns in PARR. However, in neoplastic proliferations of lymphoid cells, lymphocytes will be clones of one another resulting in similar CDR3 lengths and by contrast, resembling monoclonal patterns. Thus, PARR examines regions of immunoglobulin genes and T-cell receptors with high sensitivity and specificity, demonstrating them through monoclonal, oligoclonal and polyclonal peaks (Keller et al., 2016).

Care must be taken since some conditions of chronic inflammation and other neoplastic disorders can resemble peaks of monoclonal/oligoclonal lymphocyte expansion (Burkhard & Bienzle, 2013). Another limitation of this technique is the lack of primer design due to phenomena such as “junctional diversity” and “cross-lineage rearrangement”, as some studies mention (Hammer et al., 2016)

PARR is also capable of determining the immunophenotype but should be mostly accompanied by other tests (immunocyto/immunohistochemistry) and clinical presentation. This is due to the already mentioned cross-lineage rearrangement phenomenon where B and T cells rearrange each other’s loci, making the results interpretation harder to conclude (Hammer et al., 2016).

A big advantage of this method is that the PCR can be performed after DNA extraction from stained or non-stained cytology and histopathology slides. This allows further examination of samples and a more accurate diagnosis (Burkhard & Bienzle, 2013).

3.4.5 Flow cytometry

Flow cytometry (FC) has proven to be a useful and growing method for diagnosing feline lymphoma. This technique can simultaneously measure parameters such as cell size, cell complexity and fluorescence characteristics, allowing pathologists to accurately understand and describe neoplastic events (Martini et al., 2018).

Through the use of many different antibodies, FC is able to identify the immunophenotype of cells. The most used markers for T-cell lineage are CD3, CD5, and CD8 and for the B-cell counterpart are CD79-alpha, CD20; CD21, and IgM/IgG. This, as stated before, has relevance in the classification of the neoplastic process in each patient. Note that one big asset of this method is that it allows for the use of several antibodies in the same sample, facilitating the sampling process (Aniołek et al., 2014). Sadly, the availability of species-specific antibodies for felines is scarce and only a few studies have been published in order to evaluate cross-reactivity with monoclonal antibodies from other species. Thus, currently, the use of flow cytometry in feline lymphoma has a lot to be developed, since most previous studies have been done in canine patients (Martini et al., 2018). Another extremely valuable aspect is the information it provides on cell maturation through antigen detection and quantification. An example of this can be the antigen CD34 which is expressed by myeloid and lymphoid precursors and then lost when these exit the bone marrow, permitting a distinction between stage V lymphoma and lymphoid leukaemia. Alongside PARR, FC can also perceive tumour clonality, but through CD molecule expression (Comazzi & Gelain, 2011; Guzera et al., 2014).

Sampling for this test includes, ideally, cell suspensions. These can be from blood samples, body cavity fluids or even FNA from tissues when placed in tubes containing Ringer´s solution buffered with serum. If immunophenotyping is necessary, a minimum quantity of $2x10^6$ cells is needed (Aniołek et al., 2014). Additionally, this method can gain even more significance in veterinary medicine because of its capacity to detect minimal residual disease (MRD). This is defined as the detection of neoplastic cells during or after treatment, using methods with increased sensitivity than just their morphological counterparts. This can better help veterinarians manage their patient's prognosis (Martini et al., 2018).

Lastly, even though FC presents a higher sensitivity and specificity among the tests previously mentioned, there is still a need for other methods like histopathology to obtain the lymphoma subtype. Furthermore, only some laboratories have the equipment necessary due to its elevated cost and lack of antibodies specific for veterinary use (Guzera et al., 2014).

3.5 **Clinical Case: Nicolau** (reference intervals present in attachments)

Patient: Nicolau is a 4-year-old Siamese cat presenting for routine examination.

- **14/02/2024**

Exam performed: Hemogram (eritrogram, leukogram, and platelet count), biochemistry exams, ionogram and serology (FIV and FeLV).

Results: The leukogram revealed leucocytosis ($23.8x10^9$/L; ↑) with neutrophilia ($15.9x10^9$/L; ↑) without left shift or toxicity and monocytosis ($1.8x10^9$/L; ↑). Even though the lymphocyte count was normal ($5.4x10^9$/L), after blood smear observation, two lymphocyte populations were observed: one composed of small and well-differentiated lymphocytes without evident nucleoli and a population of medium lymphocytes presenting a moderate amount of basophilic cytoplasm, round to irregular nucleus and evident peripheral nucleolus. The ionogram was unremarkable. The patient also tested negative for FeLV antigen and was inconclusive for FIV antibodies on ELISA.

Observations: The medium lymphocyte population was reported to be possibly reactive, not excluding the possibility of maybe being neoplastic. Further investigation for lymphadenomegaly or enlarged organs was advised as it was not possible to completely rule out neoplasia only through the hemogram/blood smear observation. Other suggested possible causes for reactive lymphocytes in younger patients were included (secondary to vaccination, chronic infection, viremia and immune-mediated disease). The inconclusive FIV test was advised to be repeated with a new fresh serum sample within 2-4 weeks.

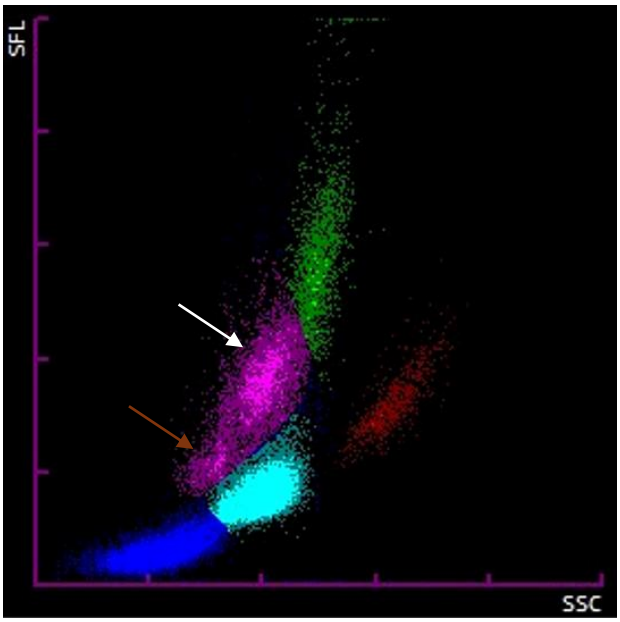


Figure 13 - Scattergram of Nicolau´s blood sample. WBC-DIFF channel scattergram showing two different populations of lymphocytes. The populations showed by both brown and white arrows have distinct quantity of nucleic acid content and cellular complexity. Axis: Y represents Side-fluorescence light (SFL) - the amount of nucleic acids and cell organelles and X represents Side-scatter light (SSC) – internal cell complexity. Obtained from Sysmex XN-V haematology analyser and based on (Brown et al., 2023)

- **24/02/2024**

Exams performed: Hemogram (eritrogram, leukogram, and platelet count), basic biochemical exams, ionogram, proteinogram, and serology for *Toxoplasma gondii* and PCR for feline *Calicivirus, Chlamydia spp., Mycoplasma haemofelis, Mycoplasma haemominutum, Mycoplasma turicensis, Bartonella henselae,* FIV provirus and feline *Coronavirus.*

Results: The leukogram revealed leucocytosis ($30.8x10^9$/L; ↑) with neutrophilia ($22.2x10^9$/L; ↑) with moderated toxicity and monocytosis($3.0x10^9$/L; ↑). The same populations of lymphocytes ($5.1x10^9$/L) were, once again, reported. The ionogram was within normal values. All serological exams were negative. The proteinogram revealed a peak in the alpha-2 fraction.

Observations: Further investigation was once again recommended. Peaks in the alpha-2 fraction usually happen in acute inflammatory reactions.

- **25/02/2024**

Exam performed: Fluid cytology of pleural effusion, stained with May-Grünwald Giemsa (MGG).

Results: The fluid was described as turbid red with a total protein count of 3.0 g/dL. Parameters such as total cell count, albumin, cholesterol and triglycerides were not evaluated due to lack of sample quantity. Cytological exam revealed a mixed cell population with the majority of cells being small mature lymphocytes. Medium forms also reported as well as macrophages and rare degenerated neutrophils without bacterial phagocytosis.

Observations: Cytology revealed a heterogenous population classified as lymphorrhagic. Due to the lack of a sample, it was not possible to evaluate if the effusion presented a chylous origin or not. The main differential diagnoses to be considered were cardiovascular diseases, small cell lymphoid neoplasia, and compression of lymphatic vessels due to non-exfoliative neoplasm or granuloma, among others. Further investigation should be performed in these cases, namely, PARR to rule out neoplasia, given the clinical and analytical history.

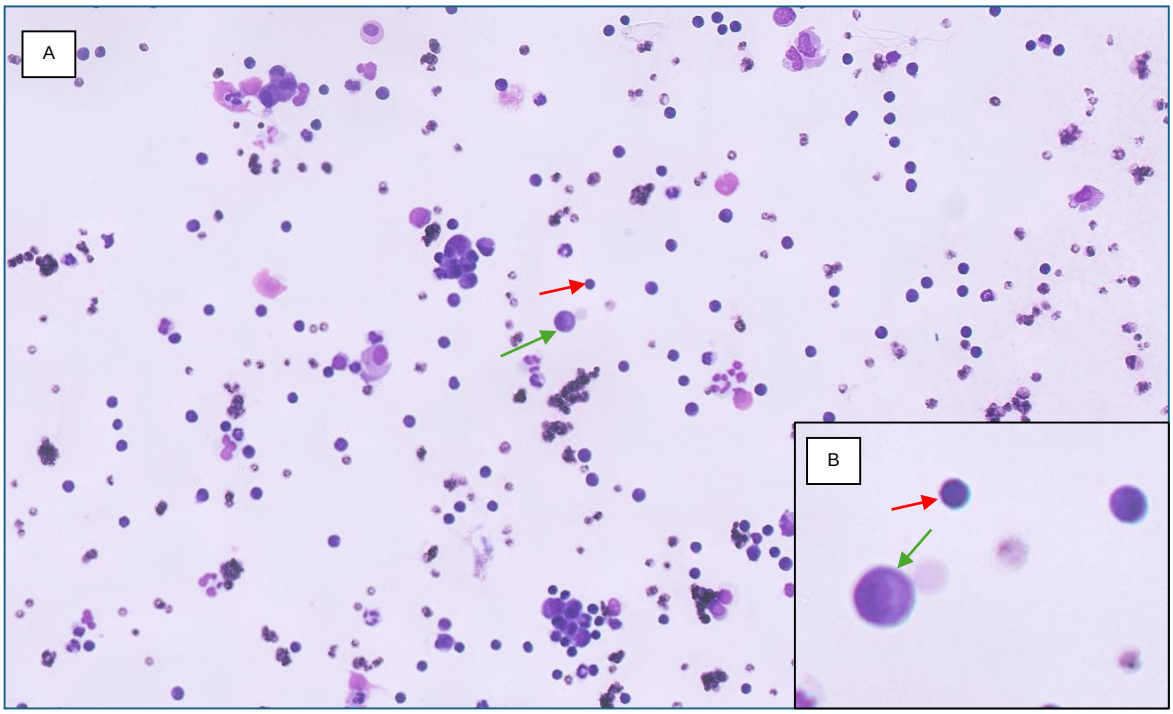


Figure 14 - Nicolau´s effusion fluid cytology. A) Intense presence of small (red arrow) and medium (green arrow) lymphocytes, macrophages and neutrophils. 20x. B) Detail to better show the different size of the lymphocyte population (small - red arrow; medium - green arrow). 40x. May-Grunwald Giemsa (MGG).

- **4/02/2024**

Exam performed: Hemogram (eritrogram, leukogram, and platelet count), biochemical exams, ionogram.

Results: The leukogram revealed leucocytosis ($33.9x10^9$/L; ↑) by neutrophilia ($27.6x10^9$/L; ↑) with left shift, toxicity and monocytosis ($1.4x10^9$/L; ↑). Lymphocyte populations ($4.2x10^9$/L) with similar features to those previously described were observed and, once again, reported. The ionogram was within normal values.

Observations: Same suggestions as the previous exam.

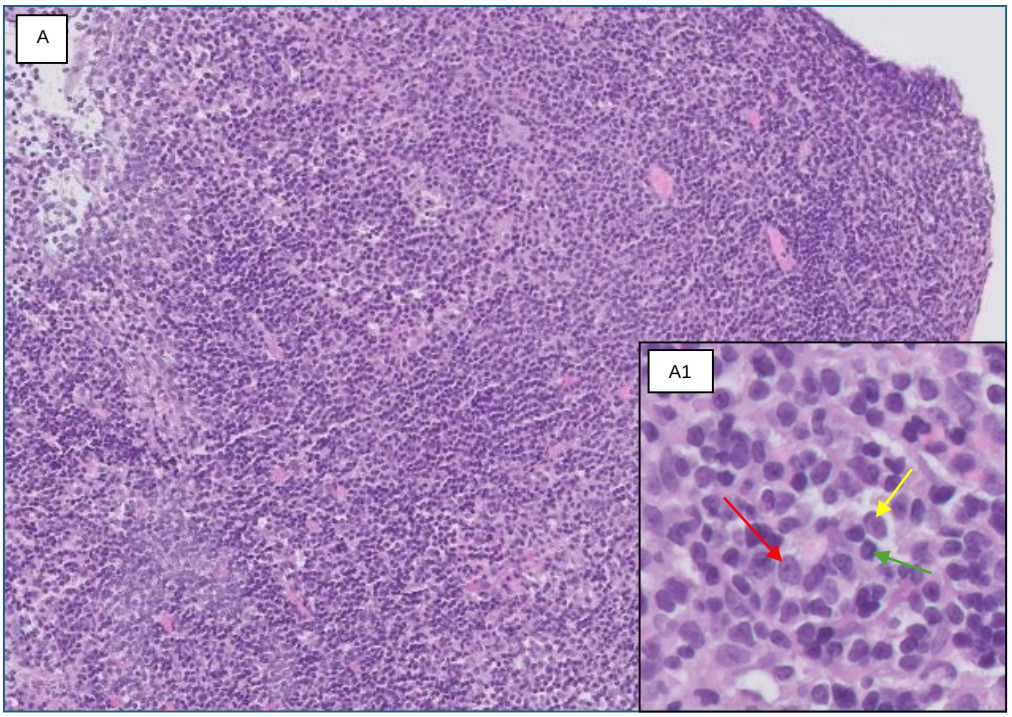
- **14/03/2024**

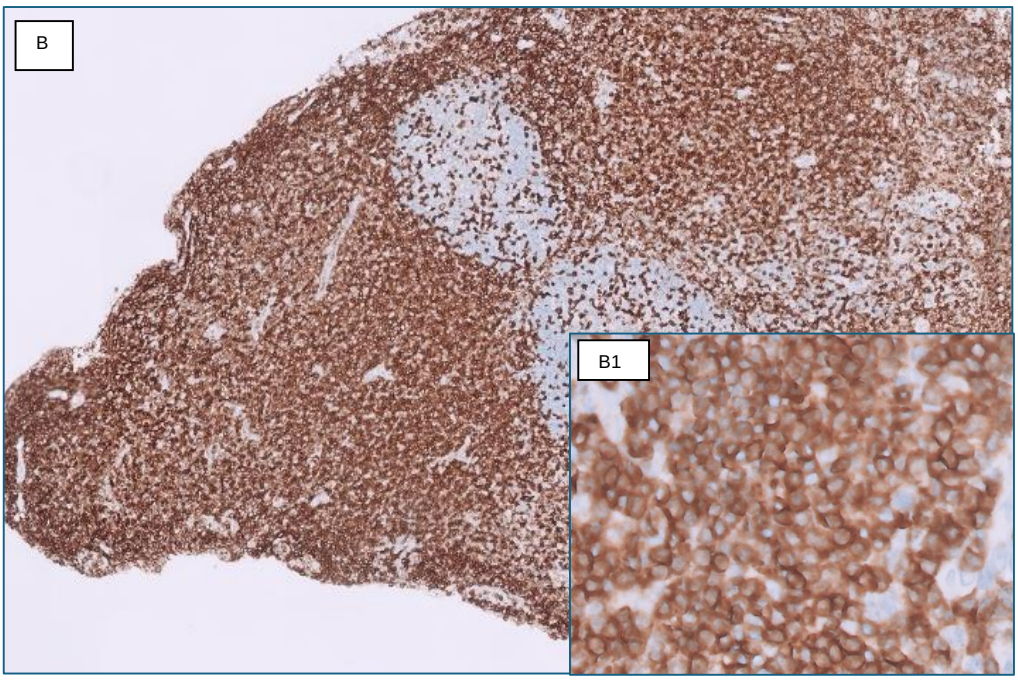
Exam performed: Histopathology by representative biopsy of mesenteric lymph node. Stained with haematoxylin and eosin (H&E).

Results: The normal lymph node normal architecture was altered due to the proliferation of a small lymphocyte population arranged in nests and supported with fibrous stroma. These cells exhibited a round to ovoid-shaped nucleus with mild pleomorphism and scarce cytoplasm. Some medium-sized lymphocytes were also identified. Prominent nucleoli was present in the lymphocytic population. Few mitosis figures were observed. In addition,

inflammatory infiltrate composed of scarce neutrophils, plasma cells and eosinophils was present.

Observations: Results were more compatible with a small cell lymphoma diagnosis. Immunohistochemistry was then performed using the markers CD3 for T-cell and PAX-5 for B-cell, showing a T-cell origin (CD3 positivity higher than 80%).





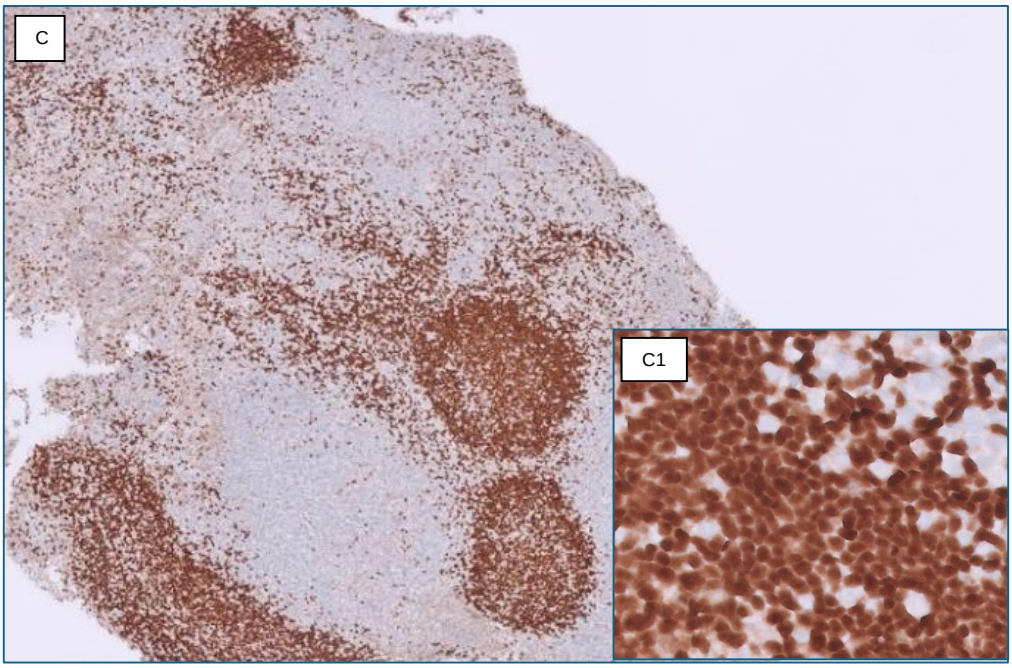


Figure 15 - Nicolau´s mesenteric lymph node microscopic evaluation. A) Intense predominance of small and medium lymphocytes that substitute the normal architecture of the organ. H&E. 10x. A1) Detail of the small (green arrow) and medium (yellow arrow) lymphocytes where most cells show evident nucleolus (red arrow). H&E. 40x. B) Immunohistochemistry with anti-CD3 showing the immunoreactivity of about 80% of the lymph node population. Immunoperoxidase-diaminobenzidine stain with Mayer's haematoxylin counterstain. 5x. B1) Inset shows membranous CD3 immunoreactivity compatible with T lymphocytes. Immunoperoxidase-diaminobenzidine stain with Mayer's haematoxylin counterstain. 40x. C) Immunohistochemistry with anti-PAX-5 showing the immunoreactivity of about 20% of the lymph node population. Immunoperoxidase-diaminobenzidine stain with Mayer's haematoxylin counterstain. 5x. C1) Inset shows nuclear PAX-5 immunoreactivity compatible with B lymphocytes. Immunoperoxidase-diaminobenzidine stain with Mayer's haematoxylin counterstain. 40x.

- **30/03/2024**

Exam performed: Hemogram (eritrogram, leukogram, and platelet count), biochemical exams, ionogram and proteinogram.

Results: The leukogram revealed leucocytosis ($26.4x10^9$/L; ↑) by neutrophilia ($19.2x10^9$/L; ↑) now without left shift or toxicity. The same lymphocytic population ($5.9x10^9$/L) was, once again, reported. The proteinogram revealed a lower peak in the alpha-2 fraction.

Observations: The lymphocyte population was now strongly considered as neoplastic. Peaks in the alpha-2 fraction usually happen in acute inflammatory reactions and, because this one was lower than the previous reported, we can assume there was an improvement in Nicolau´s pro-inflammatory state.

3.6 Discussion and the role of Clinical Pathology

Nicolau had an interesting course of events that led to a more compatible diagnosis of small-cell lymphoma. Right from the start, analysis of blood sample through flow cytometry revealed two populations of lymphocytes with different amounts of nucleic acids, cell organelles and nuclear complexity. This would be later confirmed through blood smear observation, where small and medium lymphocytic cells were described. Small mature lymphocytes are expected in circulation, but not those more immature and medium-sized ones which are assumed to be reactive or neoplastic. When neoplastic, these cells can represent cases of advanced-stage lymphoma or leukaemia, hence the importance of further investigation for their origin (Harvey, 2013; Vail et al., 2019). With these differentials, lymphocytosis could be expected, though this is not always the case (Gabor et al., 2006). This observed population would later be clarified based on histopathological findings.

In the present case, the presence of neutrophilia and monocytosis, along with possibly reactive lymphoid cells, could reveal an acute inflammatory response and might have been the cause for testing against several agents. In diseases such as infectious feline peritonitis (FIP), this type of inflammatory response is commonly seen in leukograms and fluid cytology of effusions (Valenciano & Cowell, 2020). On the other hand, in lymphoma cases, it is somehow expected to have neutrophilia and monocytosis (Gabor et al., 2006) which is why the hemogram by itself cannot be solely used for diagnosis. Additionally, care must be taken in cases like this since these changes can also reflect an eventual stress leukogram (though it is usually accompanied by lymphopenia) seen in animals subjected to endogenous/exogenous cortisol (e.g. chronic illness) (Stockham & Scott, 2013; eClinPath, 2013). Nevertheless, this last assumption seemed the less probable scenario.

The exclusion of FeLV and FIV was of major importance in Nicolau´s case due to its more compatible lymphoma diagnosis, as both of these retroviral diseases are reported to be highly related with lymphoid neoplasia development. Thus, making it mandatory to perform these tests since they end up having prognostic value in cats (Mason, S., & Pittaway, C. , 2022; Twomey, 2005).

Proteinogram changes in Nicolau's cause were mild. Despite this, there was still an improvement from the first to the second analysis (with a lower peak in the alpha-2 fraction, which is normally increased in acute inflammatory states). It can then be assumed as an improvement in Nicolau´s pro-inflammatory state (Stockham & Scott, 2013). Other infectious and/or inflammatory causes could have been ruled out to better understand what was leading to the observed graphic changes. From the available literature, it is known that proteinograms can be useful in the diagnosis of neoplastic processes such as lymphoma (when it's a globulin-

secreting lymphoma) or plasma cell tumours in cats through monoclonal peaks in the beta and gamma fractions, even though they don't need to be present for the neoplastic process to occur (Taylor et al., 2010). A fact that might explain the absence of these peaks, in this case, is the fact that Nicolau has a T-cell lymphoma and usually the B-cell kind is the one producing immunoglobulins and causing major changes in proteinogram graphics (Gerou-ferriani et al., 2011).

Fluid cytological examination of the pleural effusion revealed a mixed cell population of small mature lymphocytes and a few medium forms of lymphocytes, macrophages and rare degenerated neutrophils without bacterial phagocytosis. With the predominant population in the effusion being lymphocytes, this could be considered a lymphorrhagic effusion. These usually result from mechanisms such as lymph stasis, lymphatic hypertension, increased lymphatic permeability, lymphatic obstruction and/or lymphatic vessel rupture (e.g. trauma). However, because of the lack of sample, triglycerides and cholesterol weren´t measured, thus impairing the correct classification (chylous or not) of this effusion. Note that, aside from the predominantly mature lymphocyte population and a white, milky appearance of plasma after centrifugation, the classification of a chylous effusion implies the assessment of parameters like TG > 100 mg/dL and Chol: TG < 1 are needed (Stockham & Scott, 2013). According to Monti & Cian (2023), the two most common cases for these types of effusions cats are cardiac disease (shoud have been investigated) or neoplasia. The presence of medium lymphocytes in the effusion similar to the ones seen in previous blood smears can raise suspicion of a neoplastic process that has already reached the bloodstream. This process could have started in the any part of the body and the cells now reached the mediastinial cavity or, another hypothesis, is that there is a neoplastic process in this cavity. Attending to these cells, cytological criteria and the effusion location, a possible differential to be could be considered is thymoma. However, no mediastinal mass was reported, plus the absence of epithelial cells (can also be absent in thymomas) and mast cells make this hypothesis also less probable (Twomey, 2005; Valenciano & Cowell, 2020). In any case, diagnostic imaging should have been used to assess for the existence of any possible mass in Nicolau´s body that could have been a primary tumour (Valenciano & Cowell, 2020).

The histopathological findings encountered in the mesenteric lymph node seem more compatible with a typical small-cell lymphoma, in which predominantly mature lymphocytes substitute the normal node architecture (Meuten, 2016). The neoplastic population was mainly positive for CD3 in about 80%, reinforcing a T-cell origin (Vail et al., 2019). In this case, due to the anatomical location, cell characteristics, immunophenotype and the literature available a feline alimentary lymphoma of T-cell origin fits a more possible final diagnosis. To better enforce

this hypothesis and reach a final answer, staging Nicolau´s case becomes mandatory. This can be done, primarily, by using diagnostic imaging on the GI tract in order to find any enlargement or abnormal mass to be then submitted to biopsy and histopathological examination. This would allow to consolidate the idea and evaluate parameters such as epitheliotropism and what tissue layers were affected (Vail et al., 2019; Burkhard & Bienzle, 2013). However, due to the existing clinical history, a nodal lymphoma cannot be fully excluded, even though it tends to be more uncommon in cats (less than 10% of cases) and affects the head and neck lymph nodes. For this reason, and once again reinforcing the idea, Nicolau would strongly benefit from methods such as ultrasound to investigate any other possible masses/enlargements present (Vail et al., 2019).

As a final note, on the haematological exams performed regarding the medium-size observed lymphocytes, these were in reduced numbers, there was no significant cytopenia alteration reported and there were not any other cells that could raise alarm. Thus, the possibility of leukaemia seems very unlikely (Vail et al., 2019).

Lastly, it would be interesting and useful to have performed PARR in both cytological and histopathological samples to better distinguish between a reactive/inflammatory population and a neoplastic one, since there were other inflammatory cells present. A monoclonal result would enforce a neoplastic origin (Hammer et al., 2016; Marsilio et al., 2023)

4. FINAL REMARKS

Clinical Pathology is a major area in veterinary medicine and, through its wide range of valences aims to contribute to a correct, precise and accurate final diagnosis. To this end, communication between pathologists and clinicians is crucial and inaccuracies or scarcity in this exchange of information will constitute a limiting factor that could compromise the work of both. The key to a successful diagnosis, eventual recovery or promotion of the animal's well-being is based on this need for cooperation and joint effort.

During this internship, I had the opportunity to learn more about the applicability and potential of Clinical Pathology in the service of veterinary diagnosis, but I also had the privilege of watching from up close the daily routine of a veterinary pathologist. Two important aspects stand out from this experience: while we are being trained, we wrongly assume that laboratory diagnosis is depersonalized and that, in the midst of machines and microscopes, we may lose focus on the animal as a patient, because in the end this action takes place backstage and not on the centre stage of the veterinary clinic. With this I realized that Clinical Pathology is an area of veterinary medicine that deals with the animal as a whole and that all this data allows us to contextualize the animal's health and disease status, allowing the veterinarian to act therapeutically in a more directed and integrated way.

Additionally, so that Pathology and Clinic can give the best and expected results, it is necessary that the pathologist and the clinician share as much information as possible about the animal. The clinical history is often decisive and the absence or scarcity makes the pathologist's action more difficult and the search for a correct diagnosis much more arduous if not impossible. Combined with clinical history, there is a need to understand the principles of each diagnostic technique, their limitations and the need to resort to various methods to exclude or confirm certain alterations. Taken together, all these conclusions enhance and give meaning to the area of Veterinary Pathology.

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ATTACHMENTS

Table 1 - Reference Intervals used at Cedivet - Labortório Veterinário. Hct – Haematocrit; Hb – Haemoglobin; MCV – Mean Corpuscular Volume; MCHC – Mean Corpuscular Haemoglobin Concentration

REFERENCE INTERVALS

PARAMETER	UNITS	DOG	CAT
RED BLOOD CELL COUNT	$x10^{12}/L$	5.4 - 8.5	5.5 - 10.0
HCT	%	37.0 - 55.0	24.0 - 46.0
HB	g/dl	12.0 - 18.0	8.2 - 15.3
MCV	fL	60.0 - 77.0	37.0 - 55.0
MCHC	g/dL	31.0 - 36.0	36.2 - 35.9
WHITE BLOOD CELL COUNT	$x10^{9}/L$	5.8 - 20.3	5.5 - 19.5
NEUTROPHIL COUNT	$x10^{9}/L$	3.7 - 13.3	2.5 - 12.5
MONOCYTE COUNT	$x10^{9}/L$	0.2 - 0.7	0.1 - 1.1
LYMPHOCYTE COUNT	$x10^{9}/L$	1.0 - 3.6	1.5 - 6.5
PLATELET COUNT – OPTIC METHOD	$x10^{9}/L$	173.0 - 486.5	156.0 - 626.0

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