UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

USE OF MINIMALLY INVASIVE TECHNIQUES IN THE DIAGNOSIS AND CHARACTERIZATION OF LYMPHOID HEMATOPOIETIC TUMORS IN DOGS

PÂMELA CRISTINA LOPES GURGEL VALENTE

Orientador: Professor Doutor António José de Freitas Duarte

Coorientadores: Professor Doutor Jorge Manuel de Jesus Correia

Professora Doutora Maria Constança Matias Ferreira Pomba

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade de Ciências Biológicas e Biomédicas

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Faculdade de Medicina Veterinária da Universidade de Lisboa, 17 de janeiro de 2025

Assinatura: Pâmela Valente

Aos Valentes,

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USO DE TÉCNICAS MINIMAMENTE INVASIVAS NO DIAGNÓSTICO E CARACTERIZAÇÃO DE TUMORES HEMATOPOIÉTICOS LINFÓIDES EM CÃES

Resumo

O linfoma é o tumor hematopoiético mais comum em cães, apresentando uma variedade de formas clínicas e morfológicas originadas em células linfóides. Em contraste, o mieloma múltiplo é considerado raro. A introdução de técnicas minimamente invasivas na medicina humana teve um impacto significativo, permitindo o diagnóstico, rastreio e monitorização precisos de neoplasias em seres humanos. O potencial de aplicação destas técnicas em animais domésticos, como cães com neoplasias hematopoiéticas, ainda carece de estudos adicionais necessários para avaliar a sua eficácia e aplicabilidade na Medicina Veterinária. O principal objetivo deste trabalho foi a implementação e utilização de técnicas de diagnóstico minimamente invasivas, como citologia, citobloco, imunocitoquímica e PCR para rearranjos de receptores de antigénios (PARR), no diagnóstico de cães com suspeita de tumores hematopoiéticos linfoides. Inicialmente, a pesquisa se concentrou em avaliar e comparar diversas técnicas minimamente invasivas utilizadas em aspirados citológicos para diagnosticar linfomas multicêntricos em 38 cães. Foi demonstrado que essas abordagens permitem diagnosticar e caracterizar parcialmente o linfoma multicêntrico em cães, sugerindo a viabilidade de obtenção do material diagnóstico necessário em uma única consulta, o que leva a ganhos significativos em termos de economia de custos e tempo, sem comprometer a precisão diagnóstica. Este trabalho reforça ainda, a importância da utilização de citoblocos no diagnóstico de neoplasias de células linfóides, demonstrando seu potencial no auxílio ao diagnóstico do mieloma múltiplo (MM) canino. Diante da hipótese de neoplasias linfóides com fenótipo plasmocitário em cães, foram realizados citoblocos de punções aspirativas de baço e medula óssea para análise imuno-coloração. Os resultados da imunofenotipagem permitiram o diagnóstico de MM e excluíram outras neoplasias linfóides. Por fim, avaliou-se a aplicabilidade das técnicas de citobloco e clonalidade no diagnóstico de um caso complexo de linfoma gastrointestinal. Neste caso clínico, o diagnóstico inicial observado pelas técnicas de citologia e citobloco de aspirados hepáticos foi sugestivo de linfoma com disseminação hepática e indicou rara co-expressão de marcadores CD3 e CD20 em linfócitos, o que foi confirmado na histopatologia com imuno-histoquímica de biópsias endoscópicas. O diagnóstico final de linfoma de células T associado à enteropatia tipo 2 com infiltração hepática foi estabelecido por PARR após a detecção do rearranjo monoclonal de células T. Estes resultados, portanto, demonstram a utilidade e acurácia de métodos minimamente invasivos no diagnóstico e caracterização de diferentes tipos de tumores hematopoiéticos linfoides em cães.

Palavras chave: Citologia, Citobloco, Clonalidade, Imunofenotipagem.

USE OF MINIMALLY INVASIVE TECHNIQUES IN THE DIAGNOSIS AND CHARACTERIZATION OF LYMPHOID HEMATOPOIETIC TUMORS IN DOGS

Abstract:

Lymphoma is the most common hematopoietic tumor in dogs, presenting a variety of clinical and morphological forms originating from lymphoid cells. In contrast, multiple myeloma is considered rare. The introduction of minimally invasive techniques in human medicine has had a significant impact, allowing for the accurate diagnosis, screening, and monitoring of neoplasms in humans. The potential for applying these techniques to domestic animals, such as dogs with hematopoietic neoplasias, still lacks the necessary additional studies to assess their efficacy and applicability in veterinary medicine. The main objective of this work was the implementation and use of minimally invasive diagnostic techniques, such as cytology, cell block, immunocytochemistry and PCR for antigen receptor rearrangements (PARR), in the diagnosis of dogs with suspected lymphoid hematopoietic tumors. Initially, the research focused on evaluating and comparing various minimally invasive techniques used in cytological aspirates to diagnose multicentric lymphomas in 38 dogs. It was shown that these approaches make it possible to partially diagnose and characterise multicentric lymphoma in dogs, suggesting the feasibility of obtaining the necessary diagnostic material in a single consultation, which leads to significant gains in terms of cost and time savings without compromising diagnostic accuracy. This work then reinforces the importance of using cell blocks in the diagnosis of lymphoid cell neoplasms by demonstrating their potential in aiding the diagnosis of canine multiple myeloma (MM). Faced with the hypothesis of lymphoid neoplasms with a plasma cell phenotype in dogs, cell blocks from aspiration punctures of the spleen and bone marrow were taken for immunohistochemical analysis. The results of the immunophenotyping allowed the diagnosis of MM and excluded other lymphoid neoplasms. Finally, the applicability of the cytoblock and clonality assays in the diagnosis of a complex case of gastrointestinal lymphoma was evaluated. In this clinical case, the initial diagnosis observed by cytology and cytoblock techniques of liver aspirates was suggestive of lymphoma with hepatic dissemination and indicated rare co-expression of CD3 and CD20 positive lymphocytes, which was confirmed in histopathology with immunohistochemistry of endoscopic biopsies. The final diagnosis of T-cell lymphoma associated with type 2 enteropathy with hepatic infiltration was established by PARR following the detection of the monoclonal T-cell rearrangement. These results, therefore, demonstrate the utility and accuracy of minimally invasive methods in diagnosing and characterizing diferents types of lymphoid hematopoietic tumors in dogs.

Keys words: Cytology, Cell block, Clonality, Immunophenotyping.

USO DE TÉCNICAS MINIMAMENTE INVASIVAS NO DIAGNÓSTICO E CARACTERIZAÇÃO DE TUMORES HEMATOPOIÉTICOS LINFÓIDES EM CÃES

Resumo alargado

Os tumores hematopoiéticos constituem um grupo complexo de doenças das células sanguíneas. Dentre estes, os tumores de origem linfoide são comumente diagnosticados em animais, apresentando características variadas conforme o tipo. Essas neoplasias incluem linfomas, leucemias linfocíticas, mielomas múltiplos, plasmocitomas e leucemia de células plasmáticas. Nos cães, o linfoma é o tipo mais comum, exibindo diversas formas clínicas e morfológicas derivadas de células linfoides, enquanto o mieloma múltiplo é relativamente raro nessa espécie.

A introdução de técnicas minimamente invasivas na medicina humana teve um impacto significativo, permitindo o diagnóstico, rastreio e monitorização de neoplasias com alta precisão. O potencial para aplicação destas técnicas em animais domésticos, como em cães com neoplasias hematopoiéticas, ainda carece de estudos necessários adicionais para avaliar sua eficácia e aplicabilidade na medicina veterinária. O principal objetivo deste trabalho foi a implementação e o uso de técnicas de diagnóstico minimamente invasivas, como a citologia, o citobloco, imunocitoquímica e PCR para rearranjos de receptores dos antigénios (PRRA), no diagnóstico de cães com suspeita de tumores hematopoiéticos linfóides, além de analisar as implicações associadas à aplicação desses métodos.

O linfoma, especialmente em sua forma multicêntrica, é considerado uma das neoplasias mais comuns na oncologia veterinária, sendo frequentemente diagnosticada em cães. Embora a histopatologia desempenhe um papel crucial no diagnóstico, prognóstico e predição do comportamento biológico dos linfomas, atualmente, os métodos minimamente invasivos têm se consolidado como alternativas cada vez mais viáveis e aplicáveis para o diagnóstico dessa doença. Inicialmente, levou-se a cabo a avaliação e comparação de diversas técnicas minimamente invasivas em aspirados citológicos para o diagnóstico de linfomas multicêntricos em cães. Um total de 38 cães, abrangendo vários sexos, idades e raças, com suspeita clínica de linfoma multicêntrico, foram avaliados. A aspiração por agulha fina foi empregada para coletar amostras de linfonodos, que foram posteriormente usadas para citologia, preparação de citobloco, PCR para rearranjo do receptor de antigénio e imunocitoquímica. Entre os animais incluídos, 31 cães receberam um diagnóstico citológico de linfoma, enquanto 7 apresentaram achados sugestivos de linfoma ou linfadenite. A imunocitoquímica em esfregaços citológicos produziu resultados inconclusivos em 50% dos casos, com 44,74% diagnosticados com linfoma de células B e 5,26% com linfoma de células T. A análise dos citoblocos identificou linfoma em 30 cães e sugeriu linfoma ou neoplasia de células redondas em 8 casos. A imunocitoquímica dos citoblocos confirmou linfoma em 35

cães, compreendendo 80% de linfomas de células B e 20% de células T. A técnica molecular de PRRA, revelou rearranjo/clonalidade monoclonal em 33 casos, com 84,85% destes sendo linfomas de células B e 15,15% de células T. Este primeiro estudo destacou a precisão das técnicas minimamente invasivas no diagnóstico e caracterização do linfoma multicêntrico em cães, reforçando sua relevância na prática clínica veterinária. Demonstrou-se que estas abordagens permitem diagnosticar e caracterizar parcialmente esta neoplasia canina, possibilitando a obtenção do material diagnóstico necessário numa única consulta. Isso pode levar a uma economia significativa de tempo e custos, sem comprometer a precisão diagnóstica.

Em seguida, avaliou-se a relevância da utilização de citoblocos na identificação de outro tipo de neoplasia hematopoiética linfóide, evidenciando seu papel crucial no diagnóstico de mielomas múltiplos caninos (MM). O diagnóstico de mieloma múltiplo em cães pode ser desafiador e complexo. O citobloco é uma técnica diagnóstica que permite a identificação e a caracterização de células neoplásicas e, portanto, podem auxiliar no diagnóstico de MM com apresentaçoes atípicas. O objetivo deste segundo trabalho foi descrever três casos clínicos nos quais os citoblocos e a imuno-histoquímica contribuíram para o diagnóstico definitivo de MM canino. Três cães, uma fêmea e dois machos, com diferentes sinais clínicos, foram apresentados para consulta com anemia, hiperproteinemia com gamopatia monoclonal e presença de plasmocitose na medula óssea. A análise citológica do baço foi realizada em dois cães e foi sugestiva da presença de linfócitos ou plasmócitos de natureza neoplásica em um dos casos e hiperplasia de plasmócitos associada à hematopoiese extramedular no outro. Dadas as hipóteses de neoplasias linfoides com fenótipo de células plasmáticas, citoblocos de punções aspirativas foram realizados para análise imuno-histoquímica com anticorpos anti-CD3, CD20, CD79αcy, PAX5 e MUM1. Os resultados revelaram coloração positiva para MUM1 em 80% das células no citoloco do baço e para CD20 e MUM1 em 70% das células nos citoblocos de medula óssea, com coloração negativa para os outros anticorpos. Os resultados da imunofenotipagem permitiram o diagnóstico de MM nos três casos e excluíram outras neoplasias linfoides possíveis. Este estudo reforçou a importância dos citoblocos no diagnóstico de diferentes neoplasias hematopoiéticas caninas, demonstrando seu potencial como ferramenta útil para o diagnóstico do mieloma múltiplo canino.

Por fim, analisou-se a aplicabilidade de técnicas minimamente invasivas, como o uso de citoblocos e ensaios de clonalidade, no diagnóstico de um caso complexo de linfoma gastrointestinal. O diagnóstico de gastroenteropatia crônica em cães pode ser difícil, e a literatura enfatiza a relevância da histopatologia aliada à imuno-histoquímica na identificação dos tipos celulares envolvidos, contribuindo para a exclusão de diagnósticos diferenciais, incluindo tipos especiais de linfomas intestinais. Entretanto, a coexpressão rara de certos imunomarcadores em células linfoides pode complicar a interpretação dos resultados. O

objetivo deste terceiro trabalho foi apresentar um caso de linfoma intestinal, com coexpressão de CD3 e CD20 em células linfoides identificadas por imunocitoquímica e imuno-histoquímica, e a necessidade de PCR para rearranjo dos receptores de antigénio para um diagnóstico preciso. O caso envolveu uma fêmea castrada da raça Labrador Retriever, de 8 anos de idade, que foi apresentada para uma consulta de segunda opinião devido a vômitos e letargia, sem resposta à terapia sintomática inicial. A análise sanguínea do animal revelou hiperbilirrubinemia e hipoalbuminemia associadas à hipocobalaminemia. A ultrassonografia abdominal identificou espessamento intestinal difuso e hepatomegalia hipoecoica. Uma aspiração por agulha fina ecoguiada do fígado foi realizada para citologia e também para imunocitoquímica do citoloco. Em seguida, biópsias gástricas e duodenais foram coletadas por gastroduodenoscopia. A citologia hepática demonstrou a presença de numerosos linfócitos, sugerindo linfoma no estágio de infiltração hepática, e a imunocitoquímica no citoloco do aspirado hepático indicou coexpressão de CD3 e CD20 nas células linfoides presentes. Já a histopatologia das biópsias gástricas e duodenais, apoiou a hipótese de linfoma gastrointestinal devido à infiltração linfoide marcada do epitélio gástrico e da mucosa intestinal, incluindo as vilosidades. A técnica de imunohistoquímica foi realizada usando anticorpos CD3, CD20, PAX5 e CD79αcy. A imunomarcação foi positiva para CD3 e CD20, que sobrepuseram populações de células linfoides, e foi negativa para todos os outros anticorpos utilizados. No teste de clonalidade, a coexpressão de linfócitos de CD3 e CD20 foi confirmada pelo rearranjo monoclonal dos receptores gama de células T. A coexpressão foi examinada em conjunto com o resultado do método molecular que detectou a presença de um rearranjo monoclonal de células T. O diagnóstico final deste caso foi de linfoma de células T associado à enteropatia tipo 2 com infiltração hepática e este foi estabelecido pelo método molecular após a deteção do rearranjo monoclonal de células T. Este trabalho sublinha a importância da combinação de métodos laboratoriais, incluindo técnicas minimamente invasivas, para o diagnóstico preciso de linfomas intestinais e ainda apresenta uma rara coexpressão de marcadores de células T e B em linfoma canino.

Os nossos resultados, portanto, demonstram a utilidade e precisão de métodos minimamente invasivos no diagnóstico e caracterização de diferentes tipos de tumores hematopoiéticos linfóides em cães.

Palavras chave: Citologia, Citobloco, Clonalidade, Imunofenotipagem.

Scientific outputs derived from this dissertation

Manuscripts

This dissertation was based on the following manuscripts:

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Valente PCLG, Peleteiro MC, Dias MJ, Vicente G, Pomba C, Duarte A, Correia J. (2024). *Multiple myeloma in dogs: Use of the cell block technique as a new diagnostic tool*. Vet Clin Pathol. Mar;53(1):93-98.
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https://doi.org/10.3390/ani12243531.

Valente PCLG, Correia J, Pomba C, Duarte A. (2024). *Lymphoma in dogs: a short review.* (manuscript in preparation)

Communications

Communications presented based on the work developed in this dissertation:

Valente, PCLG.; Peleteiro, MC; Pissarra, H; Vicente, G; Correia, J; Pomba, C; Duarte, A. *Minimally invasive methods in the diagnostic and characterization of canine multicentric lymphoma*. Poster presentation at the Congress of European Society of Veterinary Clinical Pathology, 2024.

Valente PCLG, Peleteiro C, Leal R, Carvalho S, Vicente G, Filipe A. *Intestinal lymphoma in a canine with double marking for T and B lymphocytes.* Poster presentation at the XVI Congresso Internacional Veterinário Montenegro, 2020. (Award for best poster communication at this congress).

Valente PCLG, Peleteiro C, Pomba C, Duarte A, Correia J, Castro AC, Dias MJ, Monteiro C, Leal R, Carvalho S. *Multiple myeloma in dogs: Use of immunocytochemistry in cytoblocks as a new diagnostic tool.* Oral Presentation at the XVI Congresso Internacional Veterinário Montenegro, 2020.

Valente P, Carvalho S, Peleteiro MC, Correia J, Duarte A, Pomba C. *Comparative study of two methods in the molecular diagnosis of lymphoma in dogs.* Poster presentation at the CIISA'S CONGRESS 2018, 2018.

Valente PCLG, Carvalho S, Correia J, Duarte A, Pomba C, Peleteiro C. *Which is the best immunocytochemistry method in the diagnosis of lymphoma: cell block or cytological smears?* Poster presentation at the 20th ESVCP/ECVCP Meeting, 2018.

Valente P, Carvalho S, Aboim C, Pereira V, Vicente G, Correia J, Duarte A, Pomba C, Peleteiro C. *Comparative study of two immunocytochemistry methods in the diagnosis of lymphoma in small animals.* Poster presentation at the XXIII Meeting of the Portuguese Society of Animal Pathology, 2018.

Valente P, Rodrigues C, Carvalho S, Belas A, Peleteiro C, Correia J, Duarte A, Pomba C. *Canine lymphoma: immunohistochemistry or PARR?* Oral Presentation at the XIV Congresso Internacional Veterinário Montenegro, 2018.

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General discussion, conclusion, limitations of the study and future perspectives

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List of Abbreviations

ALP - alkaline phosphatase
ALT - alanine aminotransferase
APTT - activated partial thromboplastin time
C – constant
CDR3 - complementarity region 3
CHOP - cyclophosphamide (C), hydroxydaunorubicin (H), Oncovin (O) and prednisone (P)
CL - canine lymphoma
CNS - central nervous system
CRT - capillary refill time
CT - computed tomography
D - diversity
DLBCL - Diffuse large B-cell lymphoma
DNA - deoxyribonucleic acid
EATL - enteropathies associated with intestinal T-cell lymphoma
FC - flow cytometry
FeLV - feline leukemia virus
FIV - feline immunodeficiency virus
FNA - fine needle aspiration
GI - gastrointestinal
HE - Hematoxylin and eosin
HEV - Veterinary Teaching Hospital
IBD - inflammatory bowel disease
ICC - Immunocytochemistry
Ig - immunoglobulin
IgH - immunoglobulin heavy chain
IHC - Immunohistochemistry
J - junction
MM - multiple myeloma
MR - magnetic resonance
MRD - minimal residual disease
MUM1 - Multiple myeloma oncogene 1
MZL – Marginal zone lymphoma
NHL - non-Hodgkin's lymphoma
PARR - polymerase chain reaction for antigen receptor rearrangement
PCR - polymerase chain reaction

PET - positron emission tomography

PPA - positive percentage agreement

PTCL-NOS - Peripheral T-cell lymphoma-not otherwise specified

REAL/WHO - Revised European American Lymphoma /World Health Organization

TCR -T cell receptors

TCRγ - T cell receptor gamma

TZL- T-zone lymphoma

V - variable (V)

WHO - World Health Organization

Chapter I

Introduction and literature review

1. General introduction

Lymphoid hematopoietic tumors encompass a diverse range of neoplasms with varying clinical and morphological presentations, such as lymphomas and multiple myelomas in dogs (Vail 2016; Vail et al. 2020).

Lymphomas (malignant lymphoma or lymphosarcoma) are among the most frequently diagnosed tumors in dogs and humans (Swerdlow et al. 2008; Zandvliet 2016) and represent approximately 80% of all hematopoietic tumors in domestic animals (Valli et al. 2017). They comprise a diverse group of neoplasms that have in common their origin in lymphocytes and are defined as a proliferation of malignant lymphoid cells that mainly affect lymphoid tissues, which include lymph nodes or solid visceral organs, such as the liver or spleen, and bone marrow; however, they can appear in almost all tissues of the body (Vail 2016; Valli et al. 2017).

These tumors are, as a general rule, classified based on maturity and the cell lineage involved (Elenitoba-Johnson and Lim 2018; Vail et al. 2020). Traditionally, human medicine divides them into Hodgkin's lymphoma (which represents about 10% of all lymphomas) and non-Hodgkin's lymphoma (NHL) (Armitage et al. 2017). Due to similar pathological, molecular, signaling and incidence characteristics, there is a large trend in the literature that supports the use of canine lymphoma as an animal model comparative to human NHL. Given this and taking into account the alignment of biological similarities and the current approach to diagnosis, one of the main classifications of canine lymphoma is based on the human guidelines of the World Health Organization (Seeling et al. 2016), currently updated to Revised European American Lymphoma /World Health Organization (REAL/WHO).

For dogs suspected of having lymphoma, the diagnostic evaluation should include a complete physical examination, clinical pathological, imaging, cytological or histological, immunophenotypic and molecular examinations (Vail et al. 2020; Zandvliet 2016). The most common anatomical form of lymphoma in dogs is the multicentric form, observed in 80% to 85% of cases and generally characterized by the presence of peripheral lymphadenopathy. Gastrointestinal lymphomas are much less common and represent 5% to 7% of all lymphomas in the species (Valli et al. 2017; Vail et al. 2020).

Although the classification of malignant lymphoma in dogs has long been based on anatomical location, histological criteria and immunophenotypic characteristics, currently, this classification has progressed through the implementation of criteria broadly related to the cytological characteristics observed for its greater differentiation (Valli et al. 2017; Vail et al. 2020). Subcategorization of the various types of lymphoma in small animals is becoming more available and may ultimately allow for more accurate prognosis and more individualized therapy (Vail 2016). The former WHO classification contributed to a growing appreciation of the potential biological scope of canine lymphoma. However, it has become evident that a

multifactorial diagnostic approach, utilizing microscopy (cytology and histology), immunophenotyping, molecular characteristics (if known), and biological behavior, is most appropriate for diagnosing the disease (Seeling et al. 2016; Valli et al. 2017; Vail et al. 2020). Therefore, although biopsy, with histopathological and immunohistochemical evaluation are sometimes recommended, they unfortunately require more invasive procedures and may take longer to obtain a final diagnosis (Martini et al. 2022). On the other hand, cytopathology is less invasive and its results are obtained more quickly, and the use of complementary less invasive techniques has been considered a good alternative in the diagnosis of canine lymphomas, mainly due to the use of samples collected by needle aspiration (Waugh et al. 2016; Ehrhart et al. 2019; Heinrich et al. 2019; Wiley et al. 2019; Marrinhas et al. 2022; Martini et al. 2022).

In contrast to lymphoma, Multiple Myeloma (MM) represents 8% of all canine hematopoietic tumors, is considered rare and represents less than 1% of all malignant tumors in animals (Valli et al. 2017; Vail et al. 2020). MM is characterized by the systemic proliferation of malignant plasma cells or their precursors, originating from a single cell clone (Vail et al. 2020). This condition typically affects multiple bone marrow sites and is associated with monoclonal gammopathy and multiple osteolytic bone lesions (Valli et al. 2017; Vail et al. 2020). The etiology of MM is for the most part unknown (Vail 2016; Valli et al. 2017; Vail et al. 2020). It primarily occurs in elderly dogs and shows no breed or sex predilection (Vail 2016).

When MM is suspected, a complete blood count, platelet count, serum biochemistry profile, and urinalysis should be performed first. Serum electrophoresis should be conducted to detect monoclonal gammopathy, and immunoelectrophoresis should be used to identify the immunoglobulin isotype involved (Vail 2016; Valli et al. 2017; Vail et al. 2020). Definitive diagnosis typically involves demonstrating plasmacytosis on bone marrow cytology, increased serum or urine myeloma proteins (M component), and detecting osteolytic bone lesions and/or other sites of visceral organ involvement. Current recommendations require more than 20% bone marrow plasmacytosis in dogs (normal ≤ 5%), with special attention to cellular atypia. A bone marrow core biopsy may be necessary (Vail 2016; Valli et al. 2017; Vail et al. 2020).

Non-invasive/minimally invasive techniques have revolutionized human oncology and have been used to improve cancer screening and diagnosis, monitoring the impact of treatment on patients over time (Wimberger et al. 2011; Beaver et al. 2014; Neoh et al. 2018). The use of these techniques in the diagnosis of neoplasms in domestic animals, as well as in humans, must be considered very relevant and requires further in-depth studies, especially in dogs with lymphoid hematopoietic neoplasms, due to their high frequency (Waugh et al. 2016; Zandvliet 2016; Martini et al. 2018; Ehrhart et al. 2019; Heinrich et al. 2019; Raskin et al. 2019; Wiley et al. 2019; Marrinhas et al. 2022; Wilson-Robles et al. 2022). In veterinary medical oncology, in the vast majority of cases, animals are treated with chemotherapy for a prolonged period of time (Zandvliet 2016, Vail et al. 2020). Low-invasive techniques such as

immunocytochemistry, liquid biopsy, cell block, cell block immunohistochemistry, polymerase chain reaction (PCR) for antigen receptor rearrangement (PARR) and flow cytometry, are now promising in veterinary medicine and have been increasingly used in the diagnosis of neoplasms in animals, including those of lymphoid origin (Waugh et al. 2016; Zandvliet 2016; Martini et al. 2018; Ehrhart et al. 2019; Heinrich et al. 2019; Raskin et al. 2019; Wiley et al. 2019; Marrinhas et al. 2022; Wilson-Robles et al. 2022).

2. Objectives

The main objective of the research presented here is to implement and utilize minimally invasive diagnostic techniques to aid in diagnosing dogs suspected of having lymphoid hematopoietic tumors.

The specific objectives of this project were the following:

- To develop, optimize, and apply immunocytochemistry, cell block, and PARR techniques to samples from dogs;

- To assess the effectiveness of these techniques in the diagnosis and characterization of diferents hematopoietic neoplasms, such as Lymphoma and Multiple Myeloma;

- To analyze the applicability, efficacy, and sensitivity/specificity of immunocytochemistry, cell blocking, and PARR techniques in the diagnosis of multicentric lymphoma in dogs, comparing them with other diagnostic methods used in routine laboratory work, aiming to obtain relevant data that assist in making quick clinical decisions, such as the initiation of appropriate treatment to these animals.

It is expected that the results obtained throughout this research will reveal greater knowledge about the applicability and potential of some minimally invasive techniques available for the accurate diagnosis of lymphoid hematopoietic tumors in dogs. Additionally, it is hoped that the acquired knowledge will be applied to the routine monitoring of dogs undergoing treatment and to the early detection of recurrences of these neoplams, with the aim of improving the survival of these animals.

3. Literature review

3.1- Introduction

Hematopoietic malignancies encompass a complex group of diseases arising from various blood cell types. These neoplasms are highly prevalent in companion dogs, constituting nearly 30% of the annual malignancies diagnosed in this population (Wilson-Robles et al. 2023). Among these, lymphoid-origin tumors are commonly diagnosed and exhibit a range of clinical features specific to each type (Vail 2016; Vail et al. 2020). These neoplasms include lymphomas, lymphocytic leukemias, multiple myelomas, plasmacytomas, and plasma cell leukemia (Vail 2016; Vail et al. 2020). This dissertation will primarily focus on multicentric lymphoma, gastrointestinal lymphoma, and multiple myeloma in dogs.

Canine lymphoma (CL) represents one of the most common lymphoid hematopoietic neoplasms observed in dogs, with a high incidence in the species and being considered the most commonly treated in veterinary oncology (Zandvliet 2016). In general, lymphomas comprise a broad category of heterogeneous neoplasms originating in lymphocytes that mainly affect the lymph nodes, spleen and liver, but they can also be considered a generic categorization of a broad and varied group of cancer subtypes that arise from lymphocytes (Vail 2016). Although CL is often viewed as a single disease, it actually comprises a series of clinically and morphologically distinct forms of neoplasms originating from lymphoid cells (Valli et al. 2011; Zandvliet 2016).

Due to the similarities observed in several aspects, canine lymphomas are comparable to human NHL, and dogs are sometimes considered a good animal models for studies of this heterogeneous family of lymphomas (Ito, D. 2014; Seeling et al. 2016; Zandvliet 2016). Based on this, one of the main approaches for the diagnosis and classification of CL is based on the human guidelines of the World Health Organization (WHO) (Valli et al. 2011; Seeling et al. 2016). According to the Revised European American Lymphoma\World Health Organization (REAL\WHO) classification, diffuse large B-cell lymphoma (DLBCL), peripheral T-cell lymphoma not otherwise specified (PTCL-NOS) and marginal zone lymphoma (MZL) are the most common subtypes in dogs. (Ito et al. 2014; Seeling et al. 2016; Valli et al. 2017). Based on anatomical location, the most common forms of CL in decreasing order of prevalence are multicentric, gastrointestinal (GI), mediastinal and cutaneous (Vail et al. 2020).

Although the annual incidence of CL is difficult to predict, as there is no broad registry of canine tumors, it is well known that this type of lymphoma represents one of the most common neoplasms observed in the species (Vail et al. 2020). Lymphoma comprises approximately 7% to 24% of all neoplasms in dogs and 80% of all canine hematopoietic

malignancies (Kaiser 1981; Valli et al. 2017; Vail et al. 2020). The majority of lymphomas in dogs are B cell, with approximately 25% to 30% being T cell (Vail 2016).

Regarding age predisposition, lymphoma can be diagnosed in dogs of any age, but middle-aged and older dogs (average age of 6 to 9 years) are considered more affected by this neoplasm, although T-cell lymphomas tend to appear in younger animals (Ernst 2016; Pinello et al. 2019). There is no apparent predisposition by sex, although intact females present a reduced risk of developing this disease (Villamil et al. 2009). In Portugal, it was recently demonstrated that the number of CL cases predominantly occurs in males (Pinello et al. 2019). Regarding breeds, although CL can affect any breed, the highest incidence is described in Boxers, Bullmastiffs, Basset Hounds, Saint Bernards, Scottish Terriers, Airedales, Pitbulls, Briards, Irish Setters, Rottweilers and Bulldogs, with Dachshunds and Pomeranians being the breeds with lower risk (Edwards et al. 2003; Ernst et al. 2016). A study in European countries, including Portugal, investigated the breed prevalence of canine lymphoma and defined that the Dobermann, Rottweiler, Boxer and Bernese mountain dog breeds demonstrated a greater and more significant predisposition to lymphoma on the continent (Comazzi et al. 2018).

Multiple myeloma is the most significant Myeloma-related disorder (MRD), due to its incidence and severity. These disorders occur when a cell from the plasma cell or immunoglobulin-producing B lymphocyte precursor lineage undergoes malignant transformation, leading to a neoplastic population of similar cells. This population is predominantly monoclonal, producing a homogeneous immunoglobulin, though biclonal and polyclonal MRD neoplasms can also occur. MRDs encompass multiple myeloma, IgM (Waldenström's) macroglobulinemia, solitary plasma-cell tumor, and immunoglobulin-secreting lymphomas and leukemias (including plasma cell leukemia) (Vail 2016; Vail et al. 2020).

Multiple myeloma is a clonal proliferation of malignant plasma cells originating in the bone marrow. It is associated with monoclonal gammopathy and multiple osteolytic bone lesions, thereby considered as a primary bone tumor (Valli et al. 2017). Although MM accounts for fewer than 1% of all malignant tumors in animals, it represents approximately 8% of all hematopoietic tumors (Matus et al. 1986; Valli et al. 2017; Vail et al. 2020). In a compilation of bone marrow disorders in 717 dogs, MM constituted 19.8% of all neoplastic processes (Weiss 2006). Older dogs are predominantly affected, with an average age between 9 and 10 years (Valli et al. 2017; Fernández and Chon 2018). Canine MMs do not present an apparent race or sex predisposition (Valli et al. 2017).

3.2- Etiology

The causes of canine lymphomas are not fully understood and are likely multifactorial. Research is investigating genetic, molecular, infectious, environmental, and immune-related

factors (Zandvliet 2016; Vail et al. 2020). Similarly, the exact cause of multiple myeloma is unclear, but potential factors include genetic predispositions, molecular changes, viral infections, chronic immune stimulation, and carcinogen exposure (Vail 2016).

Advances in molecular cytogenetic studies have been and are being applied to investigations of chromosomal aberrations in animals with lymphoma as well as in humans. Assessment of cytogenetic abnormalities can aid in the diagnosis of tumors as well as provide a more accurate prognosis for the specific mutations present (Devitt et al. 2009). Several genetic and molecular aberrations have already been documented in dogs and cats with lymphoma, but their clinical and therapeutic relevance is still being investigated (Thomas et al. 2001; Aricò et al. 2014; Vail 2016; Vail et al. 2020).

In other animal species, the existence of a virus causing a specific leukemia has already been observed, which makes possible a viral factor for CL as well. Although some types of lymphoma in cats have been associated with viral diseases such as feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV), the hypothesis that a retrovirus may be involved in the pathogenesis of lymphoma in dogs has been researched and has not been confirmed (Vail 2016). Studies with the Epstein-Barr virus, a virus involved in the etiology of some forms of lymphoma in humans, were also carried out at CL, however, there was no association between serological or molecular detection of this virus and the development of lymphoma in dogs (Milman el al. 2011; Waugh et al. 2016; Zandvliet 2016; Vail et al. 2020).

Despite the association of *Helicobacter pylori* infections with the development of lymphoma in the gastric mucosa of humans, experimental infections in Beagle dogs demonstrated only the formation of lymphoid follicles without progression to gastric lymphoma (Rossi et al. 2000). Recently, a causal role of the intestinal microbiota in the spread of different diseases has been reported, including in some tumors (Gavazza et al. 2018; Mahiddine et al. 2022). When evaluating the intestinal microbiome of dogs with lymphoma, significant differences were observed from dogs in the control group, although a cause-effect relationship was not clear (Gavazza et al. 2018; Mahiddine et al. 2022).

Several environmental factors are investigated as risk factors for the development of CL, such as the use of pesticides, waste management, exposure to tobacco, among others (Marconato et al. 2009; Takashima-Uebelhoer et al. 2012; Pinello et al. 2017). In humans, it is considered that a dysfunctional immune system may also play a role in lymphomagenesis, therefore immunosuppression, autoimmune diseases or immunodeficiency disorders may increase the risk of developing lymphoma (Zandvliet 2016). Immune system alterations, such as immune-mediated thrombocytopenia, regardless of age and sex, have been associated with a greater risk of the dog developing lymphoma later, comparing to the normal population (Keller 1992; Foster et al. 2000). One study reports the development of lymphoma in a dog following immunosuppressive therapy with cyclosporine (Blackwood et al. 2004). Another one

also suggests an association between the immunodysregulation observed in dogs with atopic dermatitis and the risk of developing epitheliotropic T-cell lymphoma (Santoro et al. 2007).

3.3- Clinical presentation

The clinical signs of canine lymphoid hematopoietic neoplasms vary depending on the type, extent, and location of the tumor. In canine lymphoma, the most common clinical presentation is the so-called multicentric form and is usually differentiated by the presence of generalized peripheral lymphadenopathy (Zandvliet 2016, Vail et al. 2020). There are also extranodal forms and include gastrointestinal, mediastinal, cutaneous, hepatic, splenic, renal, ocular, central nervous system, pulmonary, osseus, among others. It is important to highlight that the presence of paraneoplastic syndromes can make the clinical presentation of lymphoma in dogs even more complicated (Zandvliet 2016). The clinical signs and clinicopathologic abnormalities in dogs with multiple myeloma are attributed to either direct tissue infiltration by neoplastic cells or associated paraneoplastic syndromes (Atherton and Mason 2022). Due to the diverse range of pathological effects associated with the disease, the signs of this tumor can vary widely (Vail et al. 2020).

Below there are the descriptions of the two most common clinical presentations of lymphomas and MM:

3.3.1- Multicentric Lymphoma

Multicentric lymphoma is the most common clinical presentation, representing approximately 80% of canine lymphomas. It is associated with the presence of superficial lymphadenopathy, usually generalized, and may involve splenic, hepatic and/or bone marrow involvement. Enlarged lymph nodes are usually painless, rubbery in texture, and inconspicuous (Vail 2016; Vail et al. 2020).

The clinical staging of multicentric lymphoma is divided into five stages and two substages, as defined by the WHO (Table 1). This lymphoma may be limited to a single lymph node (stage I), or be present in multiple lymph nodes in one region of the body (stage II). It may also present a generalized non-painful lymphadenopathy (stage III), or have secondary involvement of the liver and/or spleen (stage IV) or blood and/or bone marrow (stage V) (Owen 1980; Zandvliet 2016). The majority of dogs with multicentric lymphoma present no major signs of systemic disease or are asymptomatic (WHO substage a); however, a variety of non-specific signs may occur, such as anorexia, weight loss, vomiting, diarrhea, ascites, dyspnea, polydipsia, polyuria, fever and hypercalcemia (WHO substage b) (Marconato et al. 2013; Zandvliet 2016; Vail et al. 2020).

Table 1: World Health Organization (WHO) clinical staging for domestic animals with lymphoma (Owen 1980).

STAGE	CRITERIA
I	Single lymph node
II	Multiple lymph nodes in a regional area
III	Generalized lymphadenopathy
IV	Liver and/or spleen involvement (with or without stage III)
V	Bone marrow or blood involvement and/or any nonlymphoid organ (with or without stages I to IV)
Substage	
A	Without clinical signs of disease
B	With clinical signs of disease (ex: fever, weight loss, hypercalcemia)

World Health Organization: TNM classification of tumors in domestic animals, Geneva, 1980, World Health Organization.

Dogs with T-cell lymphoma are more likely to have symptomatic lymphoma (i.e., substage b). Polydipsia and polyuria are often evident in dogs with paraneoplastic hypercalcemia. Some dogs present changes in blood cells secondary to marked tumor infiltration of the bone marrow (myelophthisis) or resulting from paraneoplastic syndrome. In addition to anemia, thrombocytopenia and neutropenia, they can also have fever, sepsis and hemorrhages (Vail 2016; Vail et al. 2020). Diffuse pulmonary infiltration, which can be detected by chest radiographic evaluation and bronchoalveolar lavage analysis, is observed in about 30% of dogs with the multicentric form and this (Yohn et al. 1994; Starrak et al. 1997).

3.3.2- Gastrointestinal Lymphoma

The alimentary form of lymphoma or gastrointestinal (GI) lymphoma is much less common than the Multicentric form. The GI lymphoma is more common in cats than in dogs and represents about 5% to 7% of all canine lymphomas (Coyle and Steinberg 2004; Gieger 2011; Vail et al. 2020). It can occur focally, but more frequently it affects multiple segments with wall thickening, narrowing of the lumen and frequent mucosal ulceration (Couto et al. 1989; Ozaki et al. 2006). Most canine gastrointestinal lymphomas appear to be primary, with involvement in descending order of frequency of the small intestines, stomach and colon. Soft to firm cream-colored masses are present in the gastrointestinal submucosa and may extend into the lumen and transmute into the serosa. Typically, several segments of the intestine are involved, and there are often metastases to regional lymph nodes and liver (Coyle and Steinberg 2004; Uzal et al. 2016).

Most dogs present nonspecific and rapidly progressive gastrointestinal clinical symptoms, such as vomiting, diarrhea, weight loss, malabsorption and lethargy (Frank et al.

2007). Physical examination findings in dogs with GI lymphoma may include ascites, poor body condition, presence of an abdominal mass, abdominal pain and thickened intestinal loops. The most common biochemical abnormality is hypoalbuminemia (occurring in 61%–80% of dogs), while hypercalcemia is uncommon (Frank et al. 2007; Rassnick et al. 2009). The majority of alimentary lymphomas in dogs are T-cell origin, are composed of large lymphocytes, and often present with epitheliotropism (Coyle and Steinberg 2004; Uzal et al. 2016).

On ultrasound examination, loss of normal stratification of the intestinal wall, increased thickness and mesenteric lymphadenopathy can be seen, suggesting a GI lymphoma (Penninck et al. 2003). This imaging test can be useful to discriminate enteritis from intestinal neoplasia, but it may also not present relevant changes in up to 25% of dogs with GI lymphoma (Frances et al. 2013). The definitive diagnosis of this neoplasm requires a gastrointestinal biopsy, but histologically it may be difficult to distinguish lymphoma from inflammatory bowel disease (IBD), and additional tests may be necessary, such as immunohistochemistry and PARR (Uzal et al. 2016; Vail et al. 2020).

3.3.3- Multiple Myeloma (MM)

Multiple myeloma is defined wich one clonal expansion of malignant plasma cells within the bone marrow and is primarily characterized by monoclonal gammopathy and the presence of osteolytic bone lesions (Valli et al. 2017). In the dog, the most common clinical signs are lethargy, weakness, anorexia, lameness as a result of bone destruction, paresis secondary to spinal cord compression, hemorrhages and changes in the central nervous system (Uzal et al. 2016; Vail 2016).

One of the main characteristics of MM is the presence of serum monoclonal gammopathy or, less commonly, biclonal gammopathy (Valli et al. 2017). Hypercalcemia may occur in the course of the disease as a result of bone lysis and polyuria, polydipsia and renal failure secondary to hypercalcemia and light chain proteinuria (Uzal et al. 2016). Hepatosplenomegaly can occur due to organ infiltration (Vail et al. 2020). A normocytic, normochromic, non-regenerative anemia is found in approximately two-thirds of dogs. This may be related to medullary infiltration (myelophthisis), blood loss caused by coagulation disorders, chronic inflammatory disease or increased destruction of erythrocytes secondary to high serum viscosity. Similar factors can lead to thrombocytopenia and leukopenia in 25% to 30% of affected dogs (Vail 2016).

Changes such as the presence of plasmacytosis in bone marrow examination, the presence of osteolytic bone lesions on radiographic examination and high levels of myeloma proteins in serum or urine are considered fundamental for the diagnosis of MM in dogs (Vail et al. 2020).

3.4- Classification of lymphomas

Several classification schemes for canine lymphoma have been developed based on the anatomical location of the disease, the clinical staging stipulated by the World Health Organization (WHO), histological/cytological/immunophenotypic evaluation and genotype. With advances in diagnostic techniques it has been possible to acquire data that can subcategorize this disease into prognostically important groups that can ultimately result in more personalized treatment recommendations (Vail 2016). However, even today, the main classifications of canine lymphoma are based on anatomical form, histology and immunophenotyping (Vail et al. 2020).

3.4.1- Anatomical classification

Lymphomas are classified according to their anatomical location and grouped according to the WHO classification (Owen 1980); each group has a characteristic clinical presentation (Table 2). In dogs, 80% to 85% of lymphoma cases are the multicentric peripheral nodal anatomical type and present III or IV WHO stage, followed by alimentary (≈7%), cutaneous (≈6%), mediastinal (≈3%) and various extranodal sites (liver, spleen, kidney, eyes, central nervous system, lung, bones, heart, nasal cavity), which appear less frequently (Vail 2016; Zandvliet 2016). However, due to the recent implementation of the updated WHO classification, which integrates findings on cellular morphology and molecular characteristics, the significance of the gross anatomical classification has decreased (Uzal et al. 2016).

Table 2: Anatomical classification of canine lymphomas according to WHO classification (Owen 1980).

Anatomic Classification	Clinical Presentation	Most common Immunophenotype
Multicentric	Bilateral and/or symmetrical peripheral lymphadenopathy; absence or presence of tumor metastasis to liver, spleen, tonsil, and bone marrow	B
Alimentary	Gut-associated lymphoid tissue involvement; single or multifocal/diffuse lesions, often with mesenteric lymphadenopathy	T
Mediastinal	Associated with an increase in craniomediastinal lymph nodes, thymus or both. Hypercalcaemia frequently occur as a paraneoplastic syndrome (10-40% of the cases).	T
Cutaneous	Multifocal or generalized skin involvement, may occur involvement of the oral mucosa and extracutaneous involvement of lymph nodes, liver, spleen or bone marrow.	T
Extranodal	Occur in any location outside the lymphatic system including eyes, central nervous system, bone, testes, bladder, heart, and nasal cavity	-

World Health Organization: TNM classification of tumors in domestic animals, Geneva, 1980, World Health Organization

3.4.2- Histological classification

Many histological systems have been developed for the categorization of NHL in humans, some of which have been widely adapted and adoptedth by veterinary pathologists to classify canine lymphomas. (Zandvliet 2016; Vail et al. 2020). Histologically, lymphoma in dogs is characterized based on several morphological criteria, including growth pattern, nuclear size, nuclear morphology (chromatin pattern, number and location of nucleoli), mitotic index, and immunophenotype (Zandvliet 2016). Based on these characteristics, CL has been classified in recent decades using the following classification schemes: Rappaport (Rappaport 1966; Teske et al. 1994), Lukes-Collins (USA) (Lukes and Collins 1974; Teske et al. 1994), KIEL (Europe) (Lennert and Mohri 1978; Teske et al. 1994), Working Formulation (Carter et al. 1986), updated Kiel (Teske et al. 1994; Fournel-Fleury et al. 2002), REAL (Harris et al. 1994) and WHO (Harris et al. 1999; Valli et al. 2011; Valli et al. 2013).

Currently, the histological classification of CL follows the REAL/WHO, which incorporates anatomical, morphological (cytology and histology) and immunophenotypic (B and T-cell immunophenotype) criteria to facilitate precise and replicable diagnosis (Table 3). According to this classification, the majority of lymphoma cases in dogs are represented by five principal subtypes: diffuse large B-cell lymphoma (54%), marginal zone (B-cell) lymphoma

(4%), peripheral T-cell lymphoma not otherwise specified (16%), T zone nodal lymphoma (14%), and T-lymphoblastic lymphoma (5%) (Valli et al. 2011).

Table 3: Summary of malignant lymphoma subtypes according to the Revised European American Lymphoma/World Health Organization (REAL/WHO); classification adapted for small animals (Valli et al. 2017).

B-cell neoplasms	T-cell and putative NK-cell neoplasms
Precursor B-cell neoplasms	**Precursor T-cell neoplasm**
Lymphoblastic leukemia/lymphoma	Lymphoblastic lymphoma (LBL)*/leukemia
Mature (peripheral) B-cell neoplasms	**Mature (peripheral) T-cell and NK-cell neoplasms**
- Chronic lymphocytic leukemia/ Small lymphocytic lymphoma	- Chronic lymphocytic leukemia (CLL)/ small cell lymphoma (SLL)
- Prolymphocytic leukemia	- Prolymphocytic leukemia
- Lymphoplasmacytic lymphoma	- Large granular lymphocytic (LGL) leukemia/lymphoma
- Plasmablastic lymphoma	- T-zone lymphoma (TZL), nodal*
- Mantle cell lymphoma (MCL)	- Intestinal T-cell lymphoma (enteropathy associated)
- Follicular lymphoma	- Hepatosplenic γδ T-cell lymphoma
- Diffuse large B-cell lymphoma (DLBLC)*	- Mycosis fungoides
- Angiocentric B-cell lymphoma (lymphomatoid granulomatous)	- Intravascular lymphoma (angiocentric)
- Marginal zone lymphoma (MZL)*	- Subcutaneous panniculitis–like T-cell lymphoma
Burkitt's lymphoma/Burkitt's cell leukemia	- Angioimmunoblastic T-cell lymphoma
Provisional entity: high-grade B-cell lymphoma Burkitt's-like	- Aggressive natural killer (NK)-cell leukemia/lymphoma
Plasma cell myeloma	- Adult T-cell lymphoma/leukemia
Plasmacytoma	- Anaplastic large cell lymphoma; cutaneous and systemic
	- Peripheral T-cell lymphoma not otherwise specified (PTCL-NOS)*,#

***** These five tumors account for approximately 80% of canine lymphomas.

Peripheral T-cell lymphomas not otherwise specified (PTCL-NOS) are those that are not presently specified to a specific subtype.

The most common feline lymphomas are enteric, large B-cell (includes T-cell-rich large B-cell lymphoma (TCRLBCL), nasal, mediastinal, and Burkitt's in some studies.

3.4.3- Immunophenotypic classification

Immunophenotyping holds an extreme importance in achieving diagnostic precision in canine lymphomas, as it is essential for defining the diagnosis of a series of lymphoma entities that are morphologically homogeneous but are phenotypically heterogeneous, such as distinguishing diffuse large B-cell lymphoma from lymphoma peripheral T-cells (Uzal et al. 2016).

In the veterinary literature, 60% to 80% of canine lymphomas are of B cell origin and 10% to 38% represent T-cell lymphomas. Together, B and T-cell lymphomas represent up to

22% and null cell tumors represent less than 5% (Teske et al. 1994; Fournel-Fleury et al. 2002; Vail 2016). Immunophenotypic classifications of canine lymphomas can be performed on paraffin-embedded samples, from tissue microarrays, on cytological samples obtained by fine needle aspiration (FNA) of lesions, or by flow cytometric analysis of cellular fluid samples and aspirates from lesions (Vail et al. 2020).

In the immunophenotyping of lymphomas of dogs and cats, the antibodies commonly used are anti-CD3 to detect T cells and anti-CD20 and anti-Pax-5 to detect B cells. Additional antibodies may be employed for lymphoma diagnosis, including anti CD79a, CD45, CD45RO, CD18, CD204, CD34, CD30, CD90, BCL-2, Ki-67, granzyme B, perforin, CD10, BCL-6, GCET1, FOXP1, CD204, MUM-1, immunoglobulin and antibodies light chain and, in frozen sections, CD4 and CD8 (Uzal et al. 2016)

Recently, the use of the PCR-based clonality assay for PARR, a molecular technique utilized to assess clonality within a population of lymphoid cells by amplifying deoxyribonucleic acid (DNA) sequences encoding variable regions of B cell or T cell antigen receptors, has also been used as an alternative proposal for the diagnosis and phenotyping of lymphomas (Thalheim et al. 2013; Waugh et al. 2016; Ehrhart et al. 2019).

3.5- Conventional diagnosis

In dogs with suspected lymphoma or MM, it is recommended that the initial conventional diagnostic evaluation include a complete physical examination, complete blood count, serum biochemical profile, ideally with ionized calcium measurement, and urinalysis (Vail 2016; Vail et al. 2020). Additionally, tests such as proteinogram and imaging, may be necessary, besides obtaining tissue and/or cytological samples for the evaluation and definition of a diagnosis (Vail 2016; Vail et al. 2020).

Common causes of lymphadenopathy and differential diagnosis of multicentric lymphoma include: infections caused by bacteria, viruses, protozoa (e.g. *Toxoplasma sp.*, *Leishmania sp*., *Erhrlichia sp*.) and fungal agents (e.g. *Blastomyces* and *Histoplasma sp*.); metastatic tumors such as mast cell tumor or carcinoma; immune-mediated diseases; hematopoietic tumors such as leukemias, MM, malignant or systemic histiocytosis (Vail 2016; Vail et al. 2020). In gastrointestinal lymphoma of dogs, possible differential diagnoses include other gastrointestinal tumors, foreign body, lymphangectasia, lymphoplasmacytic enteritis, systemic mycosis and gastroduodenal ulceration (Vail 2016; Vail et al. 2020).

In MM, the most common are other diseases that may be associated with monoclonal gammopathies, including lymphoid tumors (lymphoma, leukemia and plasmacytoma), chronic infections (e.g., ehrlichiosis, leishmaniasis) and monoclonal gammopathy of unknown significance (MGUS) (Vail 2016, Valli et al. 2017; Vail et al. 2020).

3.5.1- Clinical pathology

Anemia is the most common hematological change related to CL and MM, and is generally classified as normocytic and normochromic (non-regenerative). However, regenerative anemias can also occur, due to associated hemorrhagic processes or immune-mediated hemolysis (Madewell 1986; Day 1996; Vail et al. 2020). Polycythemia, described as increased red blood cell count, has been reported in renal lymphomas, possibly due to inadequate secretion of erythropoietin (Durno et al., 2011) and has also been described in dogs with MM (Ricci et al. 2021).

Thrombocytopenia occurs in 30% to 50% of CL cases, however bleeding is rarely a clinical problem (Vail et al. 2020). Although the leukocyte count is normally within the reference values for the species, both leukocytosis and leukopenia have been described (Zandvliet 2016). Neutrophilia occurs in 25% to 40% of dogs with lymphoma and lymphocytosis occurs in approximately 20% of them (Vail et al. 2020). If there is significant myelophthisis, anemia may be accompanied by thrombocytopenia and leukopenia. In dogs with MM, anemia, thrombocytopenia, and leukopenia are often present (Vail 2016; Vail et al. 2020). In multicentric lymphomas, the presence of thrombocytopenia or circulating neoplastic lymphocytes is considered suggestive of bone marrow involvement, but it does not provide conclusive evidence (Graff et al. 2014). The observation of circulating atypical lymphocytes may be indicative of stage V lymphoma or primary leukemia, and it is important to differentiate the two, given that the prognosis of each neoplastic process may vary (Vail et al. 2020).

In biochemical analyses, in dogs with high total protein concentrations or with evidence of an increased globulin fraction, serum protein electrophoresis can be performed. Monoclonal gammopathies can occur in cases of B-cell lymphoma (MacEwen and Hurvitz 1977; Zandvliet 2016). In MM, 95% of dogs have monoclonal and 5% biclonal gammopathies (Vail et al. 2020). Hypoproteinemia with hypoalbuminemia are common in gastrointestinal lymphomas (Gieger 2011; Sogame et al. 2018). Serum biochemical abnormalities generally reflect the anatomy of the site involved, as well as paraneoplastic syndromes. Hypercalcemia is documented in approximately 10% to 15% of CL cases and is almost commonly associated with T-cell lymphoma (Zandvliet 2016), and can also be seen in approximately one-third of dogs with MM (Vail et al. 2020). Increases in liver-specific enzyme activities and bilirubin concentrations or renal values may result from neoplastic infiltration in these organs, but are often secondary causes (Zandvliet 2016; Vail et al. 2020).

On urinalysis, proteinuria appears to be a common finding in dogs with multicentric lymphoma, being typically mild, independent of (sub)stage and has no impact on prognosis (Di Bella et al. 2013). In canine MM, Bence Jones (light-chain) proteinuria is present in about 45% of reported cases (Vail et al. 2020). Abnormalities in their hemostatic profile are also described in dogs with these neoplasms (Kol et al. 2015; Vail 2016).

A bone marrow aspirate or core biopsy may be recommended for complete staging, prognosis, or definitive diagnosis of CL and may be indicated in dogs with anemia, lymphocytosis, circulating atypical lymphocytes, or other cytopenias (Vail 2016; Valli et al. 2017; Vail et al. 2020). Although the presence of lymphocytes with morphological changes in the circulation of dogs with lymphoma may indicate bone marrow involvement in CL, it is important to remember that these cells can also be observed in immune-mediated and inflammatory/infectious diseases (Vail et al. 2020).

The diagnosis of canine MM requires the presence of neoplastic cells in the bone marrow, as well as serum or urinary monoclonal proteins or osteolytic lesions. For the diagnosis, it is suggested to identify more than 20% of plasma-cell density present in the cytological examination of the bone marrow aspirate, being the presence of up to 5% considered normal in the species (Valli et al. 2017).

3.5.2- Imaging

Routine thoracic and abdominal radiographs are recommended in suspected cases of lymphoid hematopoietic tumors (Vail et al. 2020).

Thoracic and abdominal radiographs are important to determine the extent of internal involvement and can frequently show irregularities in dogs with multicentric lymphoma, although these are usually nonspecific and may only suggest neoplasia as a possible differential diagnosis (Blackwood et al.1997; Geyer et al. 2010). Chest radiographs can reveal abnormal findings in about 70% of CL cases, including thoracic lymphopathy, pulmonary infiltrates, and the presence of a cranial mediastinal mass (Starrak et al. 1997). Pleural effusion fluid may also be present. Abdominal radiographs reveal evidence of involvement of the medial iliac and/or mesenteric lymph nodes, spleen or liver in approximately 50% of cases (Vail et al. 2020). In canine MM, skeletal radiographs are recommended to determine the presence and extent of osteolytic lesions, which may have diagnostic, prognostic, and therapeutic implications (Vail 2016).

Abdominal ultrasound is useful for accurately assessing lymph node size and architecture, as well as hepatic and/or splenic involvement (Nyman et al. 2006; Crabtree et al. 2010). It may also be crucial obtaining ultrasound-guided intra-abdominal specimens for diagnosis if more peripheral lesions are not evident (e.g., GI, abdominal and hepatosplenic nodal lymphoma) or if clinical staging is required (Kinns and Mai 2007). In GI lymphoma, findings such as loss of normal stratification of the intestinal wall, increased thickness and mesenteric lymphadenopathy may suggest the presence of the neoplastic process (Penninck et al. 2003). In MM, abdominal ultrasonography is recommended mainly in cats, because this modality can reveal involvement of one or more abdominal organs. This includes

splenomegaly with or without nodules, diffuse hyperechoic hepatomegaly with or without nodules, nomengaly, and enlargement of iliac lymph nodes (Vail et al. 2020).

Recently, advanced imaging modalities, including computed tomography (CT), magnetic resonance (MR), positron emission tomography (PET), or PET/CT and PET/MR imaging, are becoming more common in veterinary practice and their usefulness in the diagnosis of lymphoid neoplasms is being investigated (Yoon et al. 2004; Lawrence et al. 2009; Jones et al. 2017; Vail et al. 2020; Auger et al. 2021).

3.5.3- Cytology, histology, immunophenotyping

Cytology is a fast, sensitive and minimally invasive technique widely used to diagnose lymphoid neoplasms. Samples are collected by fine needle aspiration, which is a relatively safe and painless procedure, allowing rapid assessment and inexpensive sampling (Sözmen et al. 2005; Blauvelt and Messick 2020). It does not require hospital admission or anesthesia for the animal (Sözmen et al. 2005; Blauvelt and Messick 2020).

In dogs and cats, cytomorphological classification divides lymphomas into low grade (small cells and low mitotic rate) or high grade (large cells and high mitotic rate), which has been shown to have prognostic importance (Ponce et al. 2010; Valli et al. 2013). Although low-grade lymphomas may allow long survival, they have a worse response to chemotherapy treatment. In contrast, high-grade lymphomas initially respond well to chemotherapy and disease remission is often achieved, but, if left untreated, they are rapidly progressive and deadly. A predominance of high-grade lymphomas is observed in dogs and cats (Valli et al. 2013).

Cytomorphological description of lymphoma by cell size for prognostic purposes is often preferred and is considered an important descriptive resource. However, this assessment is no longer considered the only predictor of the biological behavior of this neoplasm, therefore, subsequent characterization by immunophenotyping, biopsy with immunohistochemistry, immunocytochemistry and/or PARR are often performed to adapt the assessment based on lymphoma classification schemes (Blauvelt and Messick 2020).

Cytological samples from lymphomas in dogs tend to be highly cellular, including cutaneous lymphomas. Lymphomas are a diverse group of lymphoid neoplasms whose main cytological characteristic is the presence of a homogeneous population of lymphocytes, but some forms include a mixed population or a subpopulation of mature lymphocytes, creating a challenge when doing the definitive cytological interpretation. Each of these neoplasms represents a clonal expansion that has distinct morphological and immunophenotypic characteristics (Valli et al. 2011; Blauvelt and Messick 2020). When immature cells make up more than 50% of the cell population, the diagnosis of malignant lymphoma can be made reliably. Generally, these neoplastic lymphocytes are larger than neutrophils and have

dispersed finely granular chromatin, nucleoli, a lower nucleus-to-cytoplasm ratio, and basophilic cytoplasm. Lymphocytes are considered medium and large if their nuclei are 1.5 to 2 times or more than 2 to 3 times the size of erythrocytes, respectively. The presence of mitosis may be more numerous than in reactive hyperplasia and macrophages may be observed. Lymphoglandular bodies are also more numerous than those in hyperplasia (Table 4) (Blauvelt and Messick 2020; Raskin 2021).

Table 4: Cytopathologic protocol and terms used to evaluate lymphoma cases (Raskin 2021).

Determine the cell size based on comparison of the nucleus to the size of an erythrocyte.
- Small: 1–1.5 × RBC diameter - Medium: 2–2.5 × RBC diameter - Large: ≥3 × RBC diameter
Determine the shape of the nucleus and its placement within the cytoplasm.
- Round: circular with no indentations - Irregularly round: few indentations or convolutions - Convoluted: several deep indentations - Clefted: single deep indentation - Central vs. eccentric placement
Determine the number, size, visibility, and location of nucleoli within the neoplastic lymphocytes.
- Single vs. Multiple - Large vs. Small - Indistinct: not visible or barely perceivable - Prominent: easily visible - Central vs. marginal or peripheral placement
Describe the cytoplasm by amount and color. Be sure to note presence of paranuclear Golgi zone or granulation.
- Scant: small rim around nucleus - Moderate size: amount intermediate between scant and abundant - Abundant: nearly twice the size of the nucleus - Pale: light in color or clear - Moderate basophilia: color intermediate between pale and dark blue - Deep basophilia: royal blue or darker
Count the total number of mitotic figures in 10 highly cellular fields under 40× objective. (If using 50× objective, count 15 fields)
- Moderate mitotic count: 3–5 mitotic figures - High mitotic count: >6 mitotic figures
Tumor grade is morphologically based on cell size and mitotic count.
- Low grade: Low mitotic count and small cell size - High grade: Moderate or high mitotic count and medium or large cell size

RBC, Red blood cell. VS, Versus.

In MM, neoplastic cells are considered to be round cells with eccentric round to oval nuclei, uniform to coarse chromatin, and a moderate amount of basophilic cytoplasm with rounded edges. Most cells have a clear perinuclear zone compatible with the Golgi apparatus. Cells that have a pink cytoplasmic border are called “flame cells”. Cells with retained cytoplasmic globules are called Mott cells. Occasionally, a pink extracellular matrix is

associated with neoplastic cells. This neoplasm can also present binucleated and multinucleated cells and variable degrees of anisocytosis and anisokaryosis. In MM, mature, small, well-differentiated plasma cells are indicators of low-grade disease, while immature, blastic-appearing plasma cells are generally characteristic of high-grade malignancy (Blauvelt and Messick 2020).

Accurate diagnosis of lymphoma requires appropriate selection and handling of tissues, and performance of ancillary tests when necessary. Ideally, a combination of cytology and histology should be performed. For the submission of tissue samples or surgical biopsies, they must be fixed in 10% buffered formalin as this allows subsequent histological assessment of tissue architecture, immunophenotyping and assessment of clonality, if necessary. Lymphoid tissue is very fragile and artifacts are commonly induced by tissue compression, delayed fixation or tissue drying. The identification of areas with good quality cellular morphology that are representative of the neoplastic process is essential, because in histopathological evaluation determining the tissue architecture, cell size and mitotic index are important parts for an accurate diagnosis of lymphoma. After differentiating a diffuse growth pattern from a follicular one, pathologists must determine the cell size. The size of neoplastic lymphoid cells in histopathological evaluation, as well as in cytological evaluation, is based on the size of lymphocyte nuclei compared to erythrocytes. The mitotic index is determined as the average number of mitotic figures in 10 random high-power fields (40×) in areas of greatest mitotic activity. A low mitotic index is defined as 0-5 mitoses, a medium mitotic index as 6-10 mitoses, and a high mitotic index as >10 mitoses (Uzal et al. 2016).

Although a definitive diagnosis of multiple myeloma usually involves only a bone marrow aspiration in dogs, a bone marrow core biopsy or multiple aspirations may be necessary due to the potential for irregular clustering or infiltration of plasma cells. In some cases, biopsies of visceral organs or osteolytic lesions may be required for diagnosis in small animals (Vail et al. 2020).

In lymphoma, immunophenotyping is used to determine the type of cells that make up the tumor, but it can also be useful in making the initial diagnosis and predicting outcome (Seeling et al. 2016; Comazzi et al. 2017; Gelain et al. 2008). When a heterogeneous population of lymphocytes is expected in a tissue, documentation of a homogeneous population of the same immunophenotype is favorable to the diagnosis of a neoplastic process. The immunophenotype of a lymphocyte is identified by determining the expression of molecules specific to B-cells (e.g., CD20) and T-cells (e.g., CD3) (Seelig et al. 2016). For accurate determination of the immunophenotype, antibodies against lymphocyte markers can be applied to tissue sections (immunohistochemistry), cytological samples (immunocytochemistry) or individual cells in a fluid medium (flow cytometry), or also through molecular diagnostic techniques (PARR). Although tumor cells sometimes have morphological

features that typify a specific immunophenotype, exceptions occur and morphological appearance cannot be used as the sole determinant of immunophenotype. Likewise, anatomical location does not always predict immunophenotype (Table 5) (Vail et al. 2020). Although immunostaining is not necessary for the diagnosis of MM, when performed, the results may vary, with the majority of cases presenting positive staining for CD79a and MUM1 and negative for CD3 (Ramos-Vara et al. 2007).

When GI lymphoma is suspected, a bowel biopsy is preferred in most cases to differentiate lymphoma from lymphocytic enteritis. If abdominal lymph nodes are involved, echo-guided punctures can be obtained with less morbidity than intestinal biopsies. Multiple samples may be needed to accurately diagnose segmental disease. Endoscopic biopsies may be inadequate given that only a superficial sample is obtained; however, deeper endoscopic biopsy techniques combined with histopathological, immunophenotypic and molecular assessments are improving the yield of this technique in diagnosing GI lymphoma (Carrasco et al. 2015; Lane et al. 2018; Sogame et al. 2018).

Table 5: Histologic and Immunophenotypic characteristics of common canine Non-Hodgkin's lymphomas in relative order of frequency (Vail et al. 2020).

Subtype	Typical Location	Histologic Architecture	Cellular Features	Immunophenotype
Diffuse large B-cell lymphoma (DLBCL)	Usually multicentric Lymphadenopathy	Diffuse	Large cells; round nuclei; one (central) or multiple nucleoli; high mitotic rate; "starry sky" appearance	CD1+, CD20+, CD21+, CD45+, CD79a+, Pax5+, MHCII+, CD18low
Peripheral T-cell lymphoma-not otherwise specified (PTCL-NOS)	Usually multicentric Lymphadenopathy	Diffuse	Variable size (small to large); irregular nuclei, variable chromatin, prominent nucleoli; varied mitotic activity	CD3+, CD79a–, CD21–, , CD45+, CD5+, CD4+/ CD8+/–, CD18high, TCRαβ
Marginal zone lymphoma (MZL)	Nodal (nMZL) or splenic (sMZL) or extranodal mucosal	Nodular/ folicular	Mostly intermediate-sized cells abundant pale cytoplasm; irregular nuclei with peripheralized chromatin and a single central nucleolus; rare mitotic figures (except nMZL)	CD1+, CD20+, CD21+, CD45+, CD79a+, MHCII+, CD18 intermediate
T-zone lymphoma (TZL)	Usually multicentric Lymphadenopathy	Nodular, paracortical, progressing to diffuse	Small to intermediate sized cells; moderate amount of pale cytoplasm; oval to elliptical nuclei with sharp, shallow indentations; nucleoli and mitotic figures are sparse	CD45–, CD3+, CD5+, CD21+, CD4+/–, CD8+/–
Precursor lymphoma	Multicentric and/or leucemia	Diffuse and/or leucemia	Intermediate-sized cells; round nuclei; scant Cytoplasm; high mitotic rate	If T-cell: CD45+, CD34+/–, CD5+/–, CD3+/–, CD4+/–, CD8–. If B-cell: CD45+, CD18+, CD34+/–, CD79a+, CD21+/–, CD20+/–

Immune staining is usually not needed for an MM diagnosis. However, when performed on cytologic and histologic preparations, most canine cases stain positive for MUM1 and CD79a and negative for CD3. CD20 is not a reliable marker for myelomas (Ramos-Vara et al. 2016; Valli et al. 2017). Additionally, CD38 and CD138 are used in humans for diagnosing this neoplasm (Ramos-Vara et al. 2016; Valli et al. 2017).

3.6- Minimally invasive techniques of diagnosis

Performing a histological examination based on tissue biopsy, whether excisional or incisional, is considered an invasive practice, despite being important for the diagnosis of some

canine and human lymphoid hematopoietic tumors (Zandvliet 2016; Huang et al. 2024). It is known, histological evaluation in these tumors, such as lymphomas, and their classification into subtypes significantly contribute to prognosis and treatment planning (Valli et al. 2011; Flood-Knapik et al. 2013). However, tissue biopsy may also not be appropriate in several situations, mainly due to the risks associated with surgical intervention and inherent limitations (Huang et al. 2024).

With the emergence of molecular diagnostics and the identification of genetic signatures associated with neoplasms, non-invasive or minimally invasive techniques have been developed to diagnose and monitor cancer in humans (Diaz and Bardelli 2014; Neoh et al. 2018). In human hematopoietic tumors, particularly lymphomas, a critical need for a minimally invasive approach has become evident, particularly in the areas of early diagnosis, prognostic monitoring, treatment response, and drug resistance (Huang et al. 2024). In veterinary medicine, there has been a growing interest in these diagnostic techniques and auxiliary tests for cytological examination, which are considered promising and whose results are comparable to the conventional methods used (Wiley et al. 2019; Melega et al. 2020, Valente et al 2024). Some minimally invasive diagnostic techniques have already been described in dogs and below, the three used in the present work will be discussed.

3.6.1- Imunocytochemistry in smears

Immunohistochemistry (IHC) is a diagnostic and research technique that relies on the affinity between antibodies and antigens to identify specific cells or molecules in formalin-fixed tissues. Immunocytochemistry (ICC), although similar to IHC, is performed specifically on cytological samples that do not have a complex architecture, including air-dried slides and cell blocks prepared from fine-needle aspirates, effusions, smears, and blood, urinary sediments and cell cultures (Camus et al. 2020). Currently, ICC is more focused on identifying specific types of cells, it is frequently used in veterinary practice in blood slides and cytological samples, especially for immunophenotyping of lymphoid neoplasms (Caniatti et al. 1996; Ramos-Vara et al. 2016; Camus et al. 2020).

The possibility of performing immunocytochemistry (ICC) on air-dried slides, obtained by minimally invasive aspiration procedures, has aroused increasing interest in this technique. With the increasing availability of commercially validated antibodies, its popularity is likely to increase further (Camus et al. 2020). Considering that two other advantages of cytology in relation to biopsy are the relatively low cost and fast response time, this scenario further reinforces the importance of cytology as a highly relevant diagnostic tool (Sözmen et al. 2005; Camus et al. 2020).

For the possible diagnosis of malignancy, conventional cytological evaluation is recommended before performing ICC. Only after cytopathological evaluation is carried out, based on Romanowsky staining, can ICC offer a more specific view of the diagnosis (Fowler and Lachar 2008). Previous reports describe the good performance of this immunophenotyping technique on stained and unstained slides (Caniatti et al. 1996; Ramos-Vara et al. 2016; Raskin et al. 2019). However, it is important to note that diagnostic laboratories generally receive a limited number of slides from each case and often most, if not all, are stained prior to evaluation by the pathologist (Raskin et al. 2019). In this context, the use of previously stained and readily available slides is highly attractive, as it can clarify the cellular origin of a neoplasm and direct the most appropriate treatment. Rapid phenotypic assessment can have a significant impact on the outcome of the diagnosis, avoiding the need to resend materials, saving time, reducing costs and minimizing patient discomfort related to more complex and invasive medical procedures (Sapierzynski et al. 2012).

In human medicine, there have been investigations into the use of various immunomarkers in phenotyping cells in previously stained slides, but few have used Romanowsky stains (Leong et al. 1999; Beraki et al. 2012). In veterinary medicine, few reports have evaluated the use of ICC on pre-stained or fixed cytological examination slides, but most studies have focused on one or two immunomarkers (Choi and Kim 2011; Sprague and Thrall 2011; Sapierzynski et al. 2012; Stone and Gan 2014; Raskin et al. 2019).

Recently, in the evaluation and optimization of ICC protocols in animals, a comparison of immunoreactivity in stained and unstained slides in blood samples, spill fluids and cytological aspirates was carried out. It was found that both approaches presented similar results and, although unstained slides produced stronger signals, this difference did not affect the diagnosis. Furthermore, it was found that ICC on material stained with a methanolic Romanowsky method can be successfully performed using antibodies against CD3ε, CD20, cytokeratin, lysozyme, Melan-A, MHCII, MUM1, Pax5 and vimentin (Raskin et al. 2019).

In a study involving canine lymphoid tumors, the joint analysis of cytological and phenotypic results obtained by immunocytochemistry on Giemsa-stained slides made it possible to identify the type and subtype of the neoplasm in 90% of the cases examined. These data are essential for planning appropriate therapy and determination of the prognosis (Sapierzynski et al. 2012). In another study, also using slides stained with Romanowsky, it was possible to characterize 49 of the 50 cases of dogs and cats with lymphoid neoplasms using anti-CD3ε, CD20 and PAX5 antibodies, with a significant association between ICC and other diagnostic tests (Raskin et al. 2019). However, the detection of signals in this technique with immunofluorescent antibodies against CD79a or CD3 has already proven to be ineffective and incapable of differentiating the cell type involved in the diagnosis of lymphoid tumors in small animals (Sawa et al. 2015).

3.6.2- Cell Block (CB)

Cell blocks consist of concentrated cytological samples, fixed and embedded in paraffin to simulate tissues obtained from pathological surgical procedures. As in conventional histological processing, these blocks are sectioned and stained according to diagnostic needs (Taylor et al. 2013; Shidham 2019). They make it possible to conduct elective complementary studies on a variety of samples, with improved cytopathological interpretation, including the opportunity to perform molecular and immunohistochemical tests (Shidham 2019). Several protocols have been described and used in animals, with more significant differences in the preparation phase. At this stage, cytological samples can be added to a variety of available materials, such as paraffin (Taylor et al. 2013; Marcos et al. 2017; Marrinhas et al. 2022), HistoGel (Joiner and Spangler 2012; Melega et al. 2020; Valente et al. 2024), surgical gel foam (Wallace et al. 2015), agarose (Zanoni et al. 2012; Fernandes et al. 2016) and commercial kits (Heinrich et al. 2019).

Although cell blocks have been used in routine human pathology for many years, they have only recently begun to be adopted in veterinary medicine. This phenomenon can be attributed to the growing interest in minimally invasive procedures and complementary tests to diagnostic cytology (Melega et al. 2020). In humans, this technique has been routinely performed on various types of samples, however, it is increasingly indicated for the majority of cytological samples (Saqi 2016; Nambirajan and Jain 2018). In dogs, its usefulness has already been described for the diagnosis of various diseases using cells isolated from matrices such as peripheral blood, cerebrospinal fluid, synovial fluid, bone marrow, cavitary effusions, urine, tissues aspirated by fine needle and bronchoalveolar lavage (Taylor et al. 2013; Wallace et al. 2015; Fernandes et al. 2016; Menezes et al. 2016; Marcos et al. 2017; Fontes Pinto et al. 2018; Haysom et al. 2018; Marcos et al. 2018; Heinrich et al. 2019; Marcos et al. 2019; Melega et al. 2020; Valente et al. 2024).

Advantages reported for cell blocks and documented in human studies include: more affordable cost; the preservation of fragile tissue architecture; the archivability of the sample due to paraffin embedding; the suitability of samples for cytochemical examinations and immunohistochemical staining; reducing the need for excisional biopsy; and the detection of certain malignancies such as lymphoma (Mayall et al. 2000; Barroca et al, 2008; Pantanowitz et al. 2010; Lynnhtun et al. 2014; Shidham 2019). Some limitations to this technique are described, including the influence of the quality of cytological samples, such as low cellularity and presence of hemodilution. The type of materials adopted in different protocols is also considered a limitation, given the possibility of artifacts occurring in the microscopic evaluation and potential interference in immunohistochemical and molecular studies (Shidham 2019; Melega et al. 2020).

Limited studies on these techniques in small animals have demonstrated their usefulness in the diagnosis of infectious and neoplastic processes, including in cases of dogs with Leishmaniasis, Multicentric and GI Lymphoma, Mesothelioma, Carcinoma and MM (Fernandes et al. 2016; Menezes et al. 2016; Heinrich et al. 2019; Milne et al. 2021; Marrinhas et al. 2022; Valente et al. 2024;). A single study carried out a systematic evaluation of the usefulness of cell blocks in diagnosing dogs with lymphadenopathy, comparing the results obtained with those of cytological examinations. The results showed a relative sensitivity of 60% for the diagnosis of lymphoma, which increased to 85% when non-diagnostic samples were excluded, and to 95% when the lymphoma and probable lymphoma interpretations were combined (Heinrich et al. 2019).

It is known that knowledge of tumor architecture and immunophenotype are necessary for CL subtyping (Flood-Knapik et al. 2013). Although ancillary immunophenotyping tests—such as immunocytochemistry (ICC), immunohistochemistry (IHC), flow cytometry (FC), and PARR—have been available for a long time, each has specific limitations. Flow cytometry (FC) is affected by sample instability, while immunohistochemistry (IHC) requires invasive sampling. PARR results may be inaccurate due to cross-lineage rearrangements, and the availability of testing centers remains limited for ICC, FC, and PARR (Mayall et al. 1997; Thalheim et al. 2013). Cell blocks are considered a validated method that combines the ease and safety of cytological aspiration puncture with the stability, archiving and broad potential for immunophenotyping, and may represent a useful complementary tool in the diagnostic evaluation of this neoplasm (Heinrich et al. 2019).

A previously developed immunohistochemical panel has been useful in differentiating between hyperplastic and neoplastic processes in canine biopsies, and this approach appears to be replicable for immunophenotyping of cell blocks (Seelig et al. 2016; Milne et al. 2021). In humans and small animals, cell block immunocytochemistry has been applied to several types of samples using conventional immunophenotyping protocols for formalin-fixed and paraffin-embedded tissues (Shidham 2019; Melega et al. 2020; Marrinhas et al. 2022; Valente et al. 2024). In dogs, some studies have explored these techniques in the diagnosis of lymphoid neoplasms. In these studies, the detection of the antigens CD3, CD20, PAX-5, MUM1, CD79a and Ki67 allowed the identification of the neoplasm, the distinction between its different types and the determination of the degree of malignancy (Fernandes et al. 2016; Heinrich et al. 2019; Marrinhas et al. 2022; Valente et al. 2024).

3.6.3- PCR for antigen receptor rearrangement (PARR)

As innovation is being adopted in veterinary medicine, molecular diagnostic tools are becoming more common. However, unlike conventional diagnostic methods, molecular tests do not have a long history of benchmarking and standardization (Ehrhart et al. 2019). Molecular

techniques have been used in the diagnosis of lymphoid neoplasms, staging, immunophenotyping and detection of minimal residual disease (MRD). Among these, the most commonly used is the PARR assay (Vernau and Moore 1999; Burnett et al. 2003; Sato et al. 2011; Thalheim et al. 2013; Aresu et al. 2014; Waugh et al. 2016; Ehrhart et al. 2019). This technique has been used in diagnosis as a way to evaluate clonality in samples when one of the differentials is CL (Waugh et al. 2016). In lymphoma, as in other neoplasms of lymphoid origin, there is a clonal expansion of lymphocytes originating from a single malignant clone; these cells share identical DNA sequences, making this clonality the hallmark of malignancy (Vail et al. 2020).

Lymphocyte differentiation depends on the process of antigen receptor rearrangement. In B and T-lymphocytes, this result is given by the recombination of variable (V), diversity (D), junction (J) and constant (C) genes, with the immunoglobulin (Ig) genes being rearranged in B-cells and the of T-cell receptors ones (TCR) on T-lymphocytes. During the process some nucleotides are broken or added between genes (within complementarity region 3 - CDR3) as they recombine, resulting in significant sequence extension and heterogeneity (Figure 1 A-B). This is unique to each lymphocyte clone and is the main determinant of antigen receptor specificity (Burnett et al. 2003; Jung et al. 2006; Langerak et al. 2012).

The PARR technique amplifies the variable regions of the Ig and TCR genes, using primers that hybridize to the conserved portions of the genes in the V and J regions, to amplify the CRD3 regions of lymphocyte DNA and, thus, detect populations clonal lesions and possible lymphoid malignancy. In lymphoid neoplasms, lymphocytes derived from the same clone have CDR3 regions of the same length and sequence, which helps in researching the clonality of these tumors (Figure 1 C) (Burnett et al. 2003; Keller et al. 2016). In general, a clonal electrophoresis profile is considered indicative of a neoplastic process, and a polyclonal electrophoresis profile is indicative of a reactive process. However, clonal results have already been described in reactive processes, in dogs with infections by *Leishmania* sp. and *Ehrlichia* sp. (Burnett et al 2003; Waugh et al. 2016; Melendez-Lazo et al. 2019; Ehrhart et al. 2019).

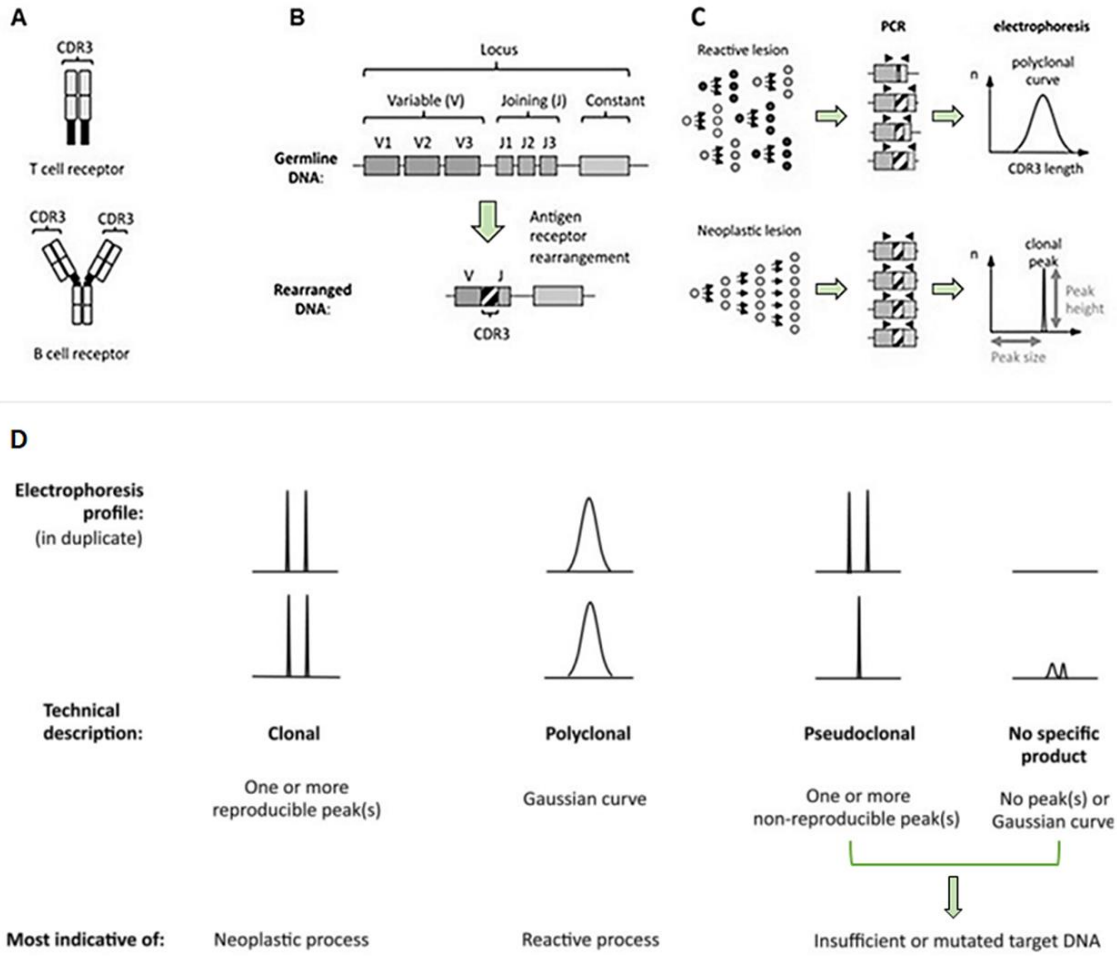

Figure 1. A- Representative image of lymphocyte antigen receptors, the T cell receptor, and the B cell receptor. B- Representative image of the gene rearrangement that occurs in the antigen receptor of lymphocytes, resulting in the formation of complementarity determining region 3 (CDR3) and encoding the antigen binding site and determines the specificity of a lymphocyte. C- Representative images of the principle of clonality testing (PARR). In reactive processes, lymphocytes are derived from several precursor cells that vary in complementarity, determining the length of region 3 (CDR3), resulting in amplicons of variable sizes and a Gaussian curve in the interpretation of capillary electrophoresis. In neoplastic processes, lymphocytes are composed of a single precursor cell and therefore have identical CDR3 lengths, resulting in equal-sized amplicons and a peak in capillary electrophoresis. D – Possible capillary electrophoresis patterns presented in PARR and interpretation of the results obtained (adapted from Keller et al. 2016).

Several sets of different primers for amplification mainly of the immunoglobulin heavy chain (IgH) and for the T-cell receptor gamma (TCRγ) genes have already been designed and improved over time in several protocols developed for the PARR technique (Vernau and Moore 1999; Burnett et al. 2003; Waugh et al. 2016; Ehrhart et al. 2019). Clonality testing is generally done on genomic DNA and can be obtained from fresh samples (fine-needle aspirates,

biopsies, blood or body fluids), fixed samples (formalin-fixed, paraffin-embedded samples or stained cytological preparations) or frozen (Lenze et al. 2012; Keller et al. 2016; Waugh et al. 2016; Ehrhart et al. 2019). Assessments of sample yield, purity and DNA integrity after extraction are recommended to reduce variations in technique and to guarantee the results obtained (Keller et al. 2016; Ehrhart et al. 2019).

PARR assays are now offered by several laboratories, mainly commercial and academic. Sensitivity and specificity values for various protocols of this technique have already been reported and are between 72% to 100% and 96% to 100%, respectively (Vernau and Moore 1999; Burnett et al. 2003; Lana et al. 2006; Tamura et al. 2006; Yagihara et al. 2007; Gentilini et al. 2009; Chaubert et al. 2010; Keller and Moore 2012; Thalheim et al. 2013; Waugh et al. 2016; Ehrhart et al. 2019). It is known that the evaluation of these results depends on the protocol used, the type of sample, cohort variability and other variables, which often cause confusion when compared (Ehrhart et al. 2019). Regarding phenotyping, 97% agreement between PARR and previous immunophenotyping is described and, although some studies suggest that this molecular method should not be used as a means of assigning cell lineage when other immunophenotyping techniques are not available or are not capable of performing adequate differentiation, the PARR assay is considered an appropriate tool for lineage determination (Waugh et al. 2016; Valente et al. 2022).

Clonality testing is increasingly used to distinguish inflammatory bowel disease from gastrointestinal (GI) lymphoma in dogs and cats and to aid in the diagnosis of splenic lymphoma, which can be difficult to distinguish from nodular hyperplasia (Gress et al. 2016; Lane et al. 2018; Ohmura et al. 2018; Sabattini et al. 2018). Molecular diagnostic techniques have received limited use to date in veterinary oncology for plasmacytoid neoplasms; However, determination of the clonality of the immunoglobulin variable region gene has been performed, using the PARR assay in feline and canine multiple plasmacytomas and myelomas. Then, the use of this technology in cases where diagnosis is not straightforward is expanding (Werner et al. 2005; Takanosu and Kagawa 2022; Wachowiak et al. 2022).

3.7 Treatment

The therapeutic approach to a given patient with lymphoma is determined by the subtype, stage and substage of the disease; presence or absence of paraneoplastic disease; the patient's overall physiological state; the financial and time commitment of the clients, and their comfort level regarding the probability of success related to treatment and/or side effects (Vail et al. 2020). Untreated dogs live an average of 4 to 6 weeks after being diagnosed with intermediate or high grade lymphoma, although significant variations can exist depending on location and subtype (Vail 2016).

In general, lymphoma is a systemic disease and requires a systemic therapeutic approach (i.e., chemotherapy, immunotherapy) to achieve remission and prolong survival. In cases of solitary or extranodal nodal lymphoma, local therapy involving surgery or radiation may be indicated (Vail 2016). Most studies related to CL therapy focus on chemotherapy treatment of intermediate- to high-grade multicentric lymphoma and information about optimal treatment. Treatment for low-grade, indolent and extranodal lymphomas is considered limited (Zandvliet 2016). Systemic multiagent chemotherapy remains the therapy of choice for intermediate and high grade canine lymphoma (Vail et al. 2020).

Several factors must be considered and discussed with animal owners when choosing a protocol for a particular situation. These factors include cost, time commitment of treatment, efficacy, toxicity, and the physician's experience with the protocols in question. In general, more complex combination chemotherapy protocols are more expensive, more time-consuming (i.e., requiring repeated office visits and closer monitoring), and more likely to result in adverse events than simpler, single-agent protocols. However, as a general rule, more complex combined protocols initially result in longer remissions and survival durations than single-agent protocols (Vail 2016).

The fundamental goals of chemotherapy for lymphoma are to induce a complete first durable (>6 months) remission (called induction), to reinduce to a remission when the tumor recurs after achieving remission (called reinduction), and finally to promote remissions when the cancer no longer responds to induction or reinduction using the initial treatment protocols (called rescue) (Vail et al. 2020). Most complex combination protocols are modifications of “CHOP,” a protocol initially designed for human oncology use. The CHOP protocol represents combinations of cyclophosphamide (C), doxorubicin (hydroxydaunorubicin [H]), vincristine (Oncovin [O]), and prednisone (P) (Vail 2016; Zandvliet 2016). The overall median remission and survival times are approximately 8 and 12 months using these protocols and approximately 20% to 25% of treated dogs are alive 2 years after starting this type of treatment (Legendre 2007; Vail et al. 2020) An example of a widely used CHOP-based induction protocol is presented in Table 6.

Table 6: University of Wisconsin–Madison: Combination chemotherapy protocol for dogs with lymphoma (Vail 2016).

Week	Drug/Dosage/Route
1	Vincristine: 0.5-0.7 mg/m^2 IV; Prednisone: 2 mg/Kg PO
2	Ciclofosfamide: 250 mg/m^2 IV***** ; Prednisone: 1.5 mg/Kg PO
3	Vincristine: 0.5-0.7 mg/m^2 IV; Prednisone: 1 mg/Kg PO
4	Doxorubicin: 30 mg/m^2 IV; Prednisone: 0.5 mg/Kg PO
5	No treatment
6	Vincristine: 0.5-0.7 mg/m^2 IV
7	Ciclofosfamide: 250 mg/m^2 IV*****
8	Vincristine: 0.5-0.7 mg/m^2 IV
9	Doxorrubicin: 30 mg/m^2 IV
10	No treatment
11	Vincristine: 0.5-0.7 mg/m^2 IV
12	Ciclofosfamide: 250 mg/m^2 IV*
13	Vincristine: 0.5-0.7 mg/m^2 IV
14	Doxorrubicin: 30 mg/m^2 IV
15	No treatment
16	Vincristine: 0.5-0.7 mg/m^2 IV
17	Ciclofosfamide: 250 mg/m^2 IV*
18	Vincristine: 0.5-0.7 mg/m^2 IV
19	Doxorrubicin: 30 mg/m^2 IV

If the patient is in complete remission at week 9, treatment continues to week 11. If the patient is in complete remission at week 19, therapy is discontinued and the dog is rechecked monthly for recurrence. A complete blood count should be performed before each chemotherapy treatment—if the neutrophil count is less than 1500 cells/mcL, the clinician should wait 5–7 days and then repeat the count; the drug is administered if the neutrophil count has risen above the 1500 cells/mcL cutoff. ***** Furosemide is given concurrently with cyclophosphamide to decrease the incidence of sterile hemorrhagic cystitis.

Rescue protocols for the treatment of CL are used in case of failure to respond to a first-line protocol or after relapse and include single-agent and multi-agent protocols. The choice of treatment protocol varies depending on the timing of relapse relative to the original protocol (first line), previously used medications (e.g., cumulative cardiotoxicity of doxorubicin), and individual physician preferences. A relapse during the first-line protocol typically requires the use of alternative drugs (i.e., drugs not included in the first protocol), while a relapse after completion of the first-line protocol leaves the possibility of inclusion of medication used in the original protocol (Zandvliet 2016). Several single agent and multiagent rescue protocols have been reported and reviewed in the veterinary literature for use in dogs (Vail et al. 2010; Parsons-Doherty et al. 2014). Rescue protocols typically result in lower response rates, shorter durations of response (2 to 3 months), and tend to show more toxicity than first-line protocols (Zandvliet 2016). The sequential application of several different rescue protocols can result in several months of prolonged survival with acceptable quality of life (Vail et al. 2020).

Most dogs with GI lymphoma present a diffuse condition, with involvement of the intestinal tract and local lymph nodes and, sometimes, the liver. Chemotherapy in dogs with diffuse medium- and high-grade disease has been considered unrewarding, with median survival times of just a few months following CHOP-based chemotherapy, and with few cases

of durable remissions reported (Frank et al. 2007; Rassnick et al. 2009; Sogame et al. 2018). Intestinal small T-cell lymphomas in dogs appear to have a more indolent course, with average survival times of 1.5 to 2.0 years being reported after conservative treatment with prednisone and chlorambucil (Couto et al. 2018; Lane et al. 2018). Solitary GI lymphomas are uncommon in dogs and can be removed surgically, depending on the location, accompanied or not by chemotherapy. Colorectal lymphoma is also usually associated with an indolent course, with overall survival sometimes greater than 3 years after the start of treatment (Desmas et al. 2017).

In canine MM, therapy is directed at both the neoplasia and secondary systemic effects. Chemotherapy is highly effective in reducing myeloma cell burden, relieving bone pain, initiating skeletal healing and reducing serum immunoglobulin levels, significantly increasing the quality and length of life for most patients. However, complete elimination of neoplastic myeloma cells is rarely achieved and eventual relapse should be expected (Vail 2016).

Two different melphalan protocols can be used to treat dogs with MM. A continuous protocol, with an initial dose of 0.1 mg/kg PO, once daily for 10 days, then reduced to 0.05 mg/kg PO, once daily continuously. A second pulse dosing protocol, a regimen that uses melphalan at a dose of 7 mg/m2 PO, daily for 5 consecutive days every 3 weeks (Fernández and Chon 2018). The addition of prednisone or prednisolone is believed to increase the effectiveness of melphalan therapy. Prednisone is initiated at a dose of 0.5 mg/kg PO once daily for 10 days and then reduced to 0.5 mg/kg every other day before discontinuation after 60 days of therapy, although some continue every other day prednisone continuously. Melphalan is continued indefinitely until clinical relapse occurs or myelosuppression necessitates dose reduction or discontinuation. The vast majority of dogs on combined melphalan and prednisone therapy tolerate the treatment well. The most clinically significant toxicity of melphalan is myelosuppression, in particular thrombocytopenia (Vail et al. 2020). Cyclophosphamide has been used as an alternative agent or in combination with melphalan in dogs and cats with MM (Gentilini et al. 2005).

3.8 Prognosis

A list of factors is known or suspected to affect remission rates and/or duration of remission and survival in dogs with lymphoma and thus the prognosis of CL. The three factors that most consistently correlate with the prognosis of dogs with lymphoma are the immunophenotypic characteristics of the tumor, the histological subtype and the WHO substage. Dogs with T-cell lymphoma generally experience significantly shorter remission and survival, although indolent T-zone lymphomas have longer survivals. The anatomical site of the disease is also considered an important prognostic factor. Lymphomas such as primary diffuse cutaneous, diffuse GI, hepatosplenic, and primary central nervous system (CNS)

lymphomas tend to be associated with a poor prognosis (Vail et al. 2020). In summary, the main factors with a strong association with worse prognosis in canine lymphoma are presented in Table 7 (Keller et al. 1993; Teske et al. 1994; Ruslander et al. 1997; Jagielski et al. 2002; Ponce et al. 2004; Marconato et al. 2011; Rao et al. 2011; Sato et al. 2011; Valli et al. 2011; Flood-Knapik et al. 2012; O'Brien et al. 2013; Valli et al. 2013; Avery et al. 2014; Seelig et al. 2014).

Table 7: Major prognostic factors for lymphoma in dogs that are strongly linked to a worse prognosis (Vail et al. 2020).

Factor	Comments
WHO clinical substage	Substage—b: associated with decreased survival.
Histopathology/Subclassification	High-grade/medium grade: associated with high response rate but reduced survival. The indolent lymphomas generally experience prolonged survivals, often in the absence of systemic therapy
Immunophenotype	T-cell phenotype associated with reduced survival (except TZL). Low MHC-II expression on B-cells associated with reduced survival.
Anatomic location	Leukemia, diffuse cutaneous and alimentary, hepatosplenic forms associated with unfavorable prognosis
Anemia	Unfavorable
Steroid pretreatment	Most reports suggest previous steroid use shortens response durations; however, length of exposure necessary is unknown.
Cranial mediastinal lymphadenopathy	Large compilation of cases reports shorter remission and survival durations

The prognosis for dogs with MM is positive for initial control and return to good quality of life. In 60 dogs with MM, >90% achieved complete or partial remission with melphalan and prednisone (Matus et al. 1986). A median survival of 540 days was reported (Vail 2016; Vail et al. 2020). Hypercalcemia, Bence Jones proteinuria and extensive bone damage are negative prognostic factors in dogs (Matus et al. 1986). Kidney disease and elevated neutrophil/lymphocyte ratio were considered negative prognostic factors (Fernández and Chon 2018). The long-term prognosis is poor due to recurrent drug resistance (Vail 2016; Vail et al. 2020).

Chapter II

Experimental Work

Comparison of the accuracy of minimally invasive techniques (cytology, cell block, immunocytochemistry and clonality assay) in the diagnosis of canine multicentric lymphoma

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Abstract

Lymphoma ranks among the most prevalent neoplasms in veterinary oncology, frequently diagnosed in dogs, particularly in its multicentric form. While histopathology plays a crucial role in lymphoma diagnosis, prognosis and prediction of biological behavior, minimally invasive diagnostic methods are increasingly emerging as viable alternatives. This study aims to assess and compare various minimally invasive diagnostic techniques for multicentric lymphomas in dogs. A total of 38 dogs, encompassing various sexes, ages, and breeds, with clinical suspicion of multicentric lymphoma, was included in the study. Fine needle aspiration was employed to collect samples from lymph nodes, which were subsequently used for cytology, cell block preparation, PCR for antigen receptor rearrangement (PARR), and immunocytochemistry. Among the animals evaluated, 31 dogs received a cytological diagnosis of lymphoma, while 7 showed findings suggestive of lymphoma or lymphadenitis. Immunocytochemistry on cytological smears yielded inconclusive results in 50% of cases, with 44.74% diagnosed with B-cell lymphoma and 5.26% with T-cell lymphoma. Cell block analysis identified lymphoma in 30 dogs and suggested lymphoma or a round cell neoplasm in 8 cases. Cell block immunocytochemistry confirmed lymphoma in 35 dogs, comprising 80% B-cell and 20% T-cell lymphomas. PARR revealed monoclonal rearrangement/clonality in 33 cases, with 84.85% of these being B-cell and 15.15% T-cell lymphomas. This study underscores the precision of minimally invasive techniques in diagnosing and characterizing multicentric lymphoma in dogs, reaffirming their significance in veterinary clinical practice.

Keys words: PARR, Diagnosis accuracy, Dog, Lymphoma, Neoplasia.

1. Introduction

Lymphoma is among the most common malignant neoplasms in dogs, encompassing many clinical and morphological subtypes. Its most frequent clinical presentation is the multicentric form, which is characterized by the presence of peripheral lymphadenopathy (Valli et al. 2011; Zandvliet 2016; Vail et al. 2020). There are several classifications described in the literature for canine lymphoma, the most commonly used being the World Health Organization (WHO) classification, based on histopathology and immunohistochemistry. Classification is highly relevant for defining clinical biological behavior, prognostic factors and response to treatment, which vary between different subtypes (Valli et al. 2011). Although biopsy and histopathological and immunohistochemical evaluation of the complete lymph node are recommended for the diagnosis of this neoplasm and frequently used in clinical practice, the use of less invasive techniques is being considered a good alternative (Zandvliet 2016; Heinrich et al. 2019; Martini et al. 2022).

Low-invasive techniques such as cytology, cell block, immunocytochemistry, polymerase chain reaction (PCR) for antigen receptor rearrangement (PARR) and flow cytometry are currently promising in veterinary medicine and have been increasingly used in the diagnosis of neoplasms in animals, including those of lymphoid origin (Waugh et al. 2016; Zandvliet 2016; Ehrhart et al. 2019; Heinrich et al. 2019; Raskin et al. 2019; Comazzi and Riondato 2021; Riondato and Comazzi 2021; Marrinhas et al. 2022; Valente et al. 2024). Flow cytometry of aspirates has been increasingly used as a first-line analysis in cases of suspected lymphoma in dogs (Comazzi and Riondato 2021; Riondato and Comazzi 2021), although access to this technique is still a limiting factor.

The simple cytological examination of a lymph node fine needle aspiration (FNA) is a fast, sensitive and non-invasive method, which makes it practically essential in the diagnosis and prognosis of canine lymphoma (Zandvliet 2016; Martini et al. 2022). Some authors have described the correlation between cytopathological and histopathological subtypes, comparing different classification systems (Martini et al. 2022). However, despite the high accuracy in the diagnosis of lymphomas, there was a lower performance of the cytological examination when additional characterization was attempted, including phenotype and grade (Martini et al. 2022). Cytology may also be inadequate for the diagnosis of low-grade lymphomas or the characterization of atypical lymphoid cells proliferations (Zandvliet 2016), reeinforcing the recommendation to perform complementary techniques to assist the diagnosis (Zandvliet 2016; Martini et al. 2022).

It is widely accepted that diagnosis of lymphoma, if based on representative FNA samples, is sufficiently reliable if sampling is enough to yield other results such as immunophenotyping and molecular tests that are cost acceptable, assist to predict prognoses,

and/ or help guide treatment options, and which will probably be applied in the future more widely than histopathology (Valli et al. 2017).

Interest in minimally invasive techniques and exams that complement cytological diagnosis has grown in recent years (Melega et al. 2020). One of these complementary techniques in the diagnosis of lymphomas is immunocytochemistry. Rapid phenotypic evaluation in cytological smears already analysed can complement the results, avoiding the need to collect additional material, eventually using more invasive procedures that may require more time to achieve a diagnosis (Sapierzynski 2012). Immunocytochemistry is routinely performed on unstained cytological smears, including blood smears, for phenotyping lymphoid neoplasms in Veterinary Medicine (Caniatti et al. 1996). However, few studies have evaluated its use in pre-stained cytological exams (Sapierzynski et al. 2012; Raskin et al. 2019). Some authors support that both stained and unstained smears can be used for this technique as they provide similar results under appropriate conditions (Raskin et al. 2019). Romanowsky-stained smears are readily available after cytological evaluation, which makes their use quite attractive, being possible to identify the cellular origin of a neoplasm (Sapierzynski et al. 2012; Raskin et al. 2019). However, this technique is not yet widely used and is not available in all veterinary laboratories.

In recent years, the cell block technique was adopted in Veterinary Medicine and applied mainly to the diagnosis of cavitary effusions and neoplastic processes (Heinrich et al. 2019; Melega et al. 2020; Marrinhas et al. 2022; Valente et al. 2024). Simple cell block microscopical observation can successfully replace lymph node smears whenever these are of poor quality due to high cell fragility or haemodilution. Some studies used immunophenotyping techniques on cell blocks of cytological samples from small animals with good results, making this practice recognized as an important tool, especially for diagnoses of neoplasms, including nodal and gastrointestinal lymphomas and multiple myelomas (Heinrich et al. 2019; Marrinhas et al. 2022; Valente et al. 2024). Several protocols are described for making these cell blocks and although they can be used with various biological samples, they have been recommended mostly for cytological samples (Shidham 2019; Melega et al. 2020).

PARR is also being used to support the diagnosis, staging and immunophenotyping of lymphomas in dogs (Zandvliet 2016). Different types of cytological samples can be analyzed with the PARR technique, mainly to assist in cases that represent a diagnostic challenge, such as those in which reactive hyperplasia and lymphoma are both considered possible, and for the categorization of the cell types involved (Vail et al. 2020). Excellent results agreement has been reported between PARR, immunohistochemistry and flow cytometry in the immunophenotyping of canine lymphomas (Waugh et al. 2016) in contrast to the findings of previous studies (Thalheim et al. 2013).

With a focus on practicality and in the best interest of animals and clients, this study aims to furnish clinicians with information that advocates for the acquisition of sufficient diagnostic material through minimally invasive techniques in the course of a single consultation.

2. Materials and methods

2.1 Sample collection

Dogs of both sexes, of various ages and breeds were selected for the present study, which, after clinical examination, were diagnosed with presumptive multicentric lymphoma, such as signs of lymphadenopathy, for example. For diagnostic confirmation one or more lymph nodes were sampled using FNA with a 10 ml syringe and a 22-gauge needle. Part of the material obtained was used to perform smears, part was placed in a microcentrifuge tube with buffered formalin to prepare the cell block and the remaining of the aspirate was placed in a dry tube for DNA extraction for the PARR technique. The samples for molecular diagnosis were immediately subjected to DNA extraction and PCR technique and, subsequently, the amplified products were subjected to capillary electrophoresis for proper interpretation. In six cases, post-mortem histopathological and immune-histochemical analyzes of lymph nodes were performed. All samples were collected for diagnostic purposes with the owners' informed consent.

All results obtained using the different techniques were provided bya minimum of two out of four veterinary pathologists, including aclinical pathologist, and reported to the responsible clinicians to assist him/her in defining an accurate diagnosis and establish a treatment protocol. A diagnostic consensus was reached among all veterinary pathologists.

2.2 Cytology

To carry out the cytological examination, FNA samples were obtained in each case from two or more lymph nodes and the smears were stained with Giemsa. The updated Kiel scheme for cytopathology was used for the evaluation of these smears as described in previous studies (Teske and van Heerde 1996; Sozmen et al. 2005). Following this, five categories were created: (i) high-grade lymphoma; (ii) low-grade lymphoma; (iii) lymphoma; (iv) suggestive of lymphoma and (v) lymphadenitis or lymphoma, similarly to what has been done by other authors (Heinrich et al. 2019).

2.3 Cell block

To prepare the cell blocks, FNA samples from lymph nodes were fixed with 10% buffered formalin in a 1.5 mL conical bottom microcentrifuge *Eppendorf* tube. After a maximum

of 24 hours fixation the cell blocks were prepared as follows. Samples were centrifuged for 5 minutes at 2800 Rpm; supernatant was discarded and the precipitate was homogenized with previously heated HistogelTM (Thermo Fisher Scientific®); samples in liquified HistogelTM were centrifuged a second time for 1 minute at 3000 Rpm. After completing the procedure, the tubes were placed in the freezer and refrigerated for 3-4 minutes to allow the Histogel cell block to solidify. The solidified block was carefully removed from the tube with the aid of a needle, cut in half and placed in a histology cassette to proceed to automated histological processing on a Leica TP1020 processor (Leica®). Following this, three micrometer sections of the paraffin block were stained with Hematoxylin & Eosin (H&E) for microscopical diagnosis and also to evaluate if the quality of the cell block justified submission to immunomarking.

2.4 Histopathology

The lymph nodes fragments collected post-mortem, properly formalin fixed, underwent automated histological processing in a Leica TP1020 tissue processor (Leica®) in accordance with the protocol routinely used at the Pathological Anatomy Laboratory. Three micrometer thick paraffin block sections were cut and stained with H&E.

2.5 Immunostaining

Cytology slides previously stained with Giemsa and cell block sections were processed in the PTLink device (Dako®) to carry out deparaffinization and/or antigen recovery steps, for a subsequent immunophenotyping process carried out according to the EnVisionTM Kit protocol (Dako, Agilent, Santa Clara, CA, USA). Anti-CD3 (polyclonal antibody, Dako, dilution 1:400) and anti-CD20 antibodies (polyclonal antibody, Biocare Medical, Pacheco, CA, USA, 1:50) were mainly used. In cases with doubtful results, anti-CD79acy (monoclonal antibody, HM57 clone, Dako, 1:200), anti Pax5 (monoclonal antibody, SP34 clone, Ventana, Tucson, AZ, USA, ready-to-use) and anti MUM1 (monoclonal antibody, BC5 clone, Biocare Medical, 1:180 dilution) antibodies were additionally used. Chromogen used was diaminobenzidine with oxygen peroxide as substrate and counterstaining performed with Mayer's hematoxylin. To ensure observer validation of this technique, positive controls were used for each antibody in all runs. All smears and histology sections from each case were processed together for immunomarking. Canine tonsil or lymph node sections are routinely used as positive controls.

2.6 PARR

DNA extraction to perform the PARR molecular technique was based on FNA lymph node samples kept in a dry eppendorf tube and sent to the laboratory, as soon as possible. Tissues DNA extraction was carried out using a commercial kit following its protocol (NZY Tissue gDNA Isolation kit - NZYtech®). After completion of the extraction, all DNA samples

were measured in the Qubit fluorometer (Invitrogen®) for fluorescence detection and then run on a 0.8% agarose gel to verify their integrity.

After DNA extraction and dosing, samples were subjected to dilutions to standardize the amount of DNA applied in each PCR reaction. Minimum concentrations between 20-30 ng of DNA per reaction were used in a total volume of 25 µl, with 250 nM of each primer and 1 x HotStarTaq Plus Master Mix (Qiagen®). Each set of primers for each reaction contained a primer with a fluorescent marker at the 5' end, using 6-carboxyfluorescein (6-FAM) or hexachlorofluorescein (HEX). The PCR protocol and criteria for interpreting the results were based on the literature (Waugh et al. 2016). Twelve pairs of primers previously described and validated for the molecular technique were used, specifically primer sets 1-10, 13 and 14 (Waugh et al. 2016). All reactions were performed using 1.5 µL of each extracted DNA and 23.5 µL of the Mix solution, containing 0.625 µL (10 µM) of each primer, 6.25µL HotStarTaq Plus Master Mix 1x (Qiagen®) and 16 µL of ultra-pure sterile water for final volume. Amplification reactions were carried out in a thermocycler (Doppio–VWR®) and consisted of an initial denaturation step of 5 minutes at 95ºC followed by 40 cycles of one minute of denaturation at 95ºC, one minute of annealing at 58ºC, one minute of extension at 72ºC, and a final extension step of 30 minutes at 72ºC, with the exception of primer sets 9, 10, 13 and 14 in which one minute of annealing at 50 ºC was used. All samples were tested in duplicate and the amplified products analyzed by capillary electrophoresis.

Mixtures for capillary electrophoresis were prepared using 1.1 µl of each PCR reaction product, added to 15 µl of a molecular weight marker dilution (GeneScan 500 ROX Size Standard, Applied Biosystems®) in ultrapure formamide (Applied Biosystems®). The PCR products amplified with primer sets 1 and 4, 2 and 5, and 3 and 6 were combined in the same mixture as they contain different fluorophores and produce fragments of different sizes. The mixtures were denatured in a thermocycler (95ºC for 3 min and rapid cooling to 4ºC). Capillary electrophoresis was performed on the 3500 Genetic Analyzer equipment (Applied Biosystems®) using a standard protocol. Analysis of capillary electrophoresis results was performed using GeneMapper 6.0 software (Applied Biosystems®). The results were presented through electropherograms, individualized according to the respective fluorophore, and Excel tables containing the molecular sizes and fluorescence intensity of the detected fragments.

2.7 Data analysis

Statistical analysis was performed using R software Version 4.1.3. The agreement between the diagnostic techniques used was calculated as positive percentage agreement (PPA), also known as relative sensitivity, with 95% confidence interval (95% CI). The term relative sensitivity is used because it is based on an imperfect reference standard method or

relative gold standard method (cytology and cell block with immunomarking) (Neta et al. 2014; Heinrich et al. 2019). All results are expressed by simple descriptive statistics, using percentages.

3. Results

Thirty-eight cases of dogs with clinical suspicion of lymphoma were evaluated, followed at the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine of the University of Lisbon, the Jamor Veterinary Center and the São Bento Veterinary Hospital. Thirty-eight dogs of different breeds were included, 16 males (42.1%) and 22 females (57.9%), aged between 4 to15 years.

Of the total dogs evaluated, 31 (81.58%) were diagnosed with lymphoma based on the cytological analysis. In six (15.79%) a diagnosis suggestive of neoplasia was issued and in one case (2.63%) the diagnosis was of lymphadenitis, although the possibility of lymphoma could not be excluded.

Out of the dogs diagnosed with lymphoma, 24 were classified as high-grade lymphoma (77.42%), five as low-grade (16.13%) and two were not classified (6.45%). Twenty cases of high-grade lymphoma were considered centroblastic (83.33%) and four lymphoblastic (16.67%). Three of the low-grade lymphomas were classified as centrocytic and two as lymphocytic. Regarding mitosis, 11 out of 31 lymphomas (35.48%) were classified as having a high mitotic count (>6 mitotic figures) (number of mitotic figures in 10 consecutive high-power fields) (Meuten et al. 2016). Three cases (9.68%) had a moderate mitotic count, and 11 cases (35.48%) had a low mitotic count. In six cases (19.36%), this parameter could not be defined.

Following microscopical examination of the cell blocks, out of the total number of suspected cases, 30 were diagnosed with lymphoma (78.95%), seven received a diagnosis of possible lymphoma (18.42%) and one of round cell neoplasia (2.63%). Immunomarking in cytology produced the following results: 19 out of the 38 cases were inconclusive (50.0%), 17 were diagnosed as type B lymphoma (44.74%) and two as type T lymphoma (5.26%). As for cell block sections immunocytochemistry, 28 were diagnosed as type B lymphoma (73.69%), seven as type T lymphoma (18.42%), one as plasma cell tumor (2.63%) and in two results were inconclusive (5.26%). In the immunohistochemistry examination of the lymph node fragments, four cases of B-cell lymphoma and two of T-cell lymphoma were defined, all results corroborating the molecular and cell block immunocytochemistry results. All cases in which the observer was unable to establish a definitive diagnosis on immunostaining were considered inconclusive. This includes samples with low cellularity in the cell block and with nonspecific staining in cytological smears, for example. Figure 2 represents the immunocytochemical diagnosis carried out in the present work, on previously Giemsa stained cytology smears and cell block sections.

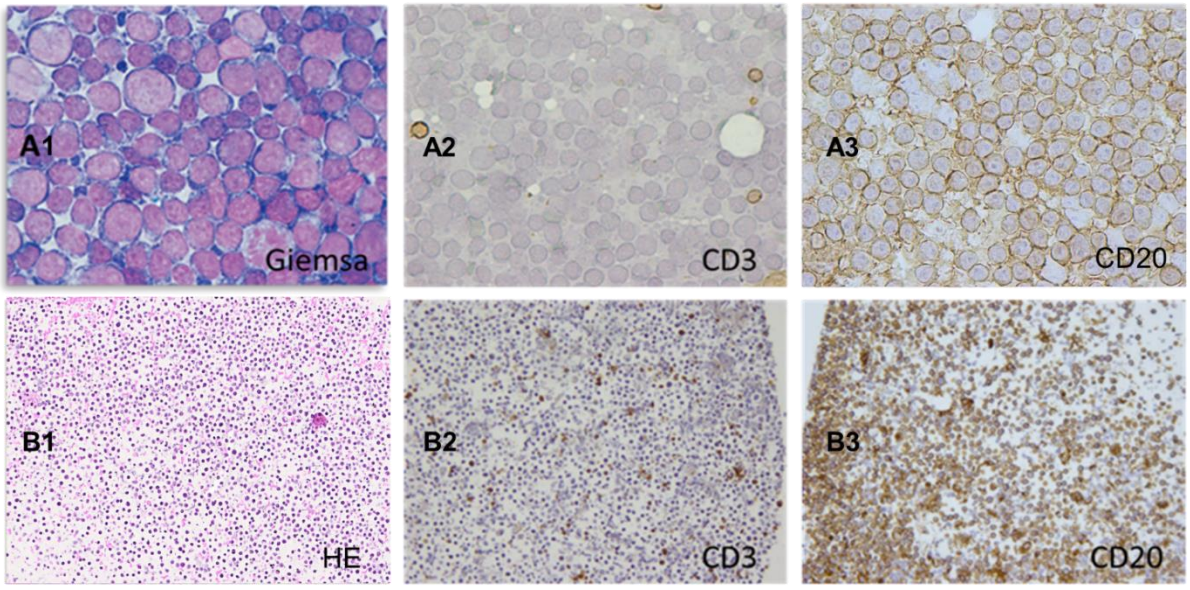

Figure 2. Immunocytochemistry applied to cytology smears and to cell blocks sections for the diagnosis of canine lymphoma. A1- Lymph node smear diagnosed as lymphoma (Giemsa, x 400); A2 - A very low number of T cells is shown (anti-CD3 antibody, diaminobenzidine chromogen, Mayer's hematoxylin, 400x); A3 - Same case showing a high percentage of B cells (anti-CD20 antibody, diaminobenzidine chromogen, Mayer's hematoxylin, 400x). B1 - Cell block section with a representative number of aspirated lymph node cells (HE, x 100); B2 A low number of T cells is shown (anti-CD3 antibody, diaminobenzidine chromogen, Mayer's hematoxylin, 100x); B3 - Same case showing a high percentage of B cells (anti-CD20 antibody, diaminobenzidine chromogen, Mayer's hematoxylin, 100x).

In the clonality PARR analysis of the 38 cases, 33 presented results compatible with monoclonal rearrangement/lymphocyte clonality/lymphoma (86.85%). In three, clonality was similar for B and T lymphocytes (7.89%) and two were considered inconclusive (5.26%). Of the cases with monoclonal rearrangement, 28 showed clonality for type B lymphocytes (84.85%) and five clonality for type T lymphocytes (15.15%). To summarize, Table 8 presents the results obtained with the diagnostic methods applied: cytology, cytology with immunostaining, cell block, cell block with immunomarking and PARR. Tables 9 and 10 presents the positive percentage agreement (PPA) or relative sensitivity of the methods used.

Table 8: Comparison of the results obtained through various diagnostic approaches in 38 cases of dog presumptive lymphoma.

Cases	Cytology	Cell block	Cytology + immunomarking	Cell block + immunomarking	PARR
1	HGL	L	I	BCL	CBC
2	LGL	L	I	TCL	CTC
3	SL	L	I	I	CBC
4	HGL	L	BCL	BCL	CBC
5	SL	L	I	TCL	CTC
6	HGL	L	BCL	BCL	CBC
7	LGL	L	BCL	BCL	CBC
8	HGL	SL	I	BCL	CBC
9	LGL	L	BCL	BCL	CBC
10	LGL	L	BCL	BCL	CBC
11	SL	SL	I	TCL	I
12	LGL	L	BCL	BCL	CBC
13	HGL	SL	I	TCL	CTC
14	HGL	SL	TCL	TCL	CTC
15	SL	L	I	BCL	CBC
16	HGL	L	I	BCL	CBC
17	SL	SL	BCL	BCL	I
18	L	L	TCL	TCL	CB/T
19	SL	RCN	I	P	CBC
20	HGL	L	BCL	BCL	CBC
21	HGL	L	I	BCL	CBC
22	HGL	L	I	BCL	CBC
23	HGL	L	I	BCL	CB/T
24	HGL	L	BCL	BCL	CBC
25	HGL	L	BCL	BCL	CBC
26	HGL	L	BCL	BCL	CBC
27	LY/L	SL	BCL	BCL	CBC
28	HGL	L	BCL	BCL	CBC
29	HGL	SL	I	TCL	CTC
30	HGL	L	I	BCL	CBC
31	HGL	L	I	BCL	CBC
32	HGL	L	BCL	BCL	CBC
33	HGL	L	I	BCL	CBC
34	HGL	L	I	BCL	CBC
35	HGL	L	BCL	BCL	CBC
36	HGL	L	BCL	BCL	CBC
37	HGL	L	I	I	CB/T
38	L	L	BCL	BCL	CBC

HGL - High grade lymphoma; **LGL** - Low grade lymphoma; **L** - Lymphoma; **SL** - Suggestive of lymphoma; **LY/L** - Lymphadenitis or Lymphoma; **I** – Inconclusive; **RCN**- Round cell neoplasm; **BCL** - B cell lymphoma; **TCL** - T cell lymphoma; **P**- Plasma cell tumor; **CBC** - Clonality for B cells; **CTC** - Clonality for T cells; **CB/T** - Clonality for B/T cells.

Table 9: Positive percentage agreement (PPA) or relative sensitivity, with 95% confidence interval (95% CI), using cytology as the relative gold standard method in the diagnosis of lymphoma.

Diagnosis of lymphoma	Cell block PPA	Nº of concordant cases	PARR PPA	Nº of concordant cases
Cytology	**90%** (73,5-97,9)	27/30	**100%** (88,8-100)	31/31

PPA – positive percentage agreement; Nº- number; Inconclusive and suggestive cases were considered negative.

Table 10: Positive percentage agreement (PPA) or relative sensitivity, with 95% confidence interval (95% CI), using cell block with immunomarking as the relative gold standard method for classification phenotypic characteristics of the lymphoma.

Phenotypic classification of lymphoma		**Cytology + immunomarking PPA**	**Nº of concordant cases**	**PARR PPA**	**Nº of concordant cases**
Cell block + immunomarking	B	61% (40,6-78,5)	17\28	92,9% (76,5-99,1)	26\28
Cell block + immunomarking	T	28,6% (3,7-71,0)	2\7	83,3% (35,9-99,6)	5\6

PPA – positive percentage agreement; B – lymphoma B; T- lymphoma T. Inconclusive and suggestive of lymphoma cases were considered negative.

4. Discussion

Although it is generally accepted that the classification of lymphomas in dogs should be based on the guidelines of the WHO, it has become evident that the most appropriate diagnostic approach must be multimodal, namely by requiring knowledge of microscopic, immunophenotypic, and clinical features before establishing a final disease diagnosis (Seelig et al. 2016).

In the first clinical evaluation of the present cases, although the clinical signs were suggestive of a neoplastic process, namely lymphoma, cytological examination refined a provisional list of differential diagnoses that included reactive hyperplasia, lymphadenitis, lymphoma and metastatic disease. This was reflected in the results of the cytological examination, with approximately 81.6% of cases diagnosed with lymphoma, mostly high grade (77.42%), even without defining the cell type involved, which demonstrates cytology as highly apropriate for the initial stages of diagnosis owing to its simplicity, low-cost, safety, and minimally-invasive sampling (Seelig et al. 2016). However, cytology does not cover important

aspects of the lymphoma classification (grade, phenotype and subtype) which require histopathology and immunohistochemistry. Currently, flow cytometry has also proven effective in diagnosing certain subtypes of lymphoma, such as low-grade/indolent lymphomas for example (Comazzi and Riondato 2021; Riondato and Comazzi 2021). Cytopathology has been considered essential in identifying lymphoma, being apropriately used as a screening test to predict the grade and phenotype, although these data must be confirmed by other complementary techniques (Martini et al. 2022). In the evaluation of 161 cytological samples of lymphoma cases performed by six examiners, the performance was excellent in diagnosing lymphoma with more than 80% agreement for all examiners (Martini et al. 2022), but consistency was much lower in the definition of grade and phenotype in particular for high grade B-cell and T-cell lymphomas. In the present study, in the 7 cases in which cytology could not define an accurate diagnosis (18.4%), the complementary techniques performed were definitive diagnosing six cases of lymphoma, four B-cell lymphomas and two T-cell lymphomas, and one case of plasmacytoma.

Currently, techniques such as cell block and PARR are increasingly being utilized in the diagnosis of lymphoid neoplasms (Waugh et al. 2016; Zandvliet 2016; Ehrhart et al. 2019; Heinrich et al. 2019; Melega et al. 2020; Marrinhas et al. 2022; Valente et al. 2024). In the present study, when compared to cytological diagnosis, they presented a relative sensitivity of 90% and 100%, respectively. In the evaluation of dogs with lymphadenopathy, a cell block technique showed 92% relative sensitivity in cases with a cytological diagnosis of lymphoma/probable lymphoma (Heinrich et al. 2019), which corroborates the results observed in the present study. Similarly, research employing PARR on cytological or histopathological samples obtained from dogs, whether fresh or fixed, demonstrated a sensitivity ranging from 85% to 100% in differentiating between lymphoma and non-lymphoma conditions (Waugh et al. 2016; Ehrhart et al. 2019). It is considered important to highlight that the quality of the sample for both techniques can significantly interfere with the results. In this study, efforts were made to minimize this issue through a rigorous cytopathological assessment of cell blocks and by collecting fresh samples for molecular analysis, performing the evaluation of DNA integrity and DNA quantification post-extraction. Although in most clinical cases the PARR technique is not performed using fresh samples, ensuring sufficient quantity and quality of DNA, is essential and must be performed on all samples analyzed. Sensitivity of PARR is highly dependent on how the assay is conducted, types and numbers of primers used, experiment conditions, interpretation guidelines and how it is validated (Waugh et al. 2016; Ehrhart et al. 2019).

Compared to the cytological examination, the cell block technique and the molecular test applied provided good results, specifically in distinguishing the cell type involved. It is important to emphasize that the agreement between the results presented may have been influenced by the number of cases analyzed in this study. Rare subtypes of lymphoma may

not have been represented in the evaluated cases, which could affect the agreement between techniques if such cases were included. In fact, in the present study, cell block with immunocytochemistry defined the largest number of conclusive diagnoses. Several other studies have supported these conclusion, as the cell block technique associated with immunophenotyping has been used for decades in human medicine (Shidham 2019) and was adopted as a technique in veterinary medicine in the diagnosis of various neoplasms in small animals (Taylor et al. 2013; Heinrich et al. 2019; Marcos et al. 2019; Marrinhas et al. 2022; Valente et al. 2024). Cell blocks play an important role by offering solid support for conducting consistent immunomarking on the same cells using various antibodies. This represents a distinct advantage of immunomarking over cytological smears (Peleteiro 2011).

Experience has also shown that cell blocks are well suitable for immunophenotyping, even when using standardized methods developed for histopathological samples (Joiner and Spangler 2012). In the present study, immunocytochemistry in smears showed worse results in the phenotyping of lymphomas when compared to cell block with immunocytochemistry, demonstrating a relative sensitivity of 61% for B lymphomas and 28.6% for T lymphomas. The elevated number of inconclusive cases observed with this technique (19 out of 38) might have occurred due to utilizing slides previously stained with Giemsa, despite this approach being described in the literature as posing no risk of failure (Sapierzynski et al. 2012; Raskin et al. 2019). Another potential factor of failure could be that the smears underwent the same technical procedures alongside histopathology sections.

The PARR molecular method, when compared to cell block with immunocytochemistry, presented a relative sensitivity of 92.9% for B lymphomas and 83.3% for T lymphomas. This molecular technique showed good performance in discriminating lymphoma from non-lymphoma, as well as in defining their phenotype, as already described (Waugh et al. 2016; Ehrhart et al. 2019). In three samples there was potential evidence of cross-lineage rearrangement. Although studies suggest that PARR should not be used as a means of assigning cell lineage due to problems with cross-lineage rearrangement (Thalheim et al. 2013), our results indicate that this occurred in a very small number of cases, as observed in other more recent works (Waugh et al. 2016; Ehrhart et al. 2019). Given this, we suggest that the use of this molecular method can be used for the phenotyping of lymphomas in small animals when other immunophenotyping techniques are not available or do not make it possible to clearly define the cell types involved, as in cases of co-expression of B and T immunomarkers in the same cells in lymphomas of dogs and cats (Granum 2015; Valente et al. 2022).

While it might be argued that our suggestion to obtain cellular material in a single visit poses a challenge in preserving DNA for subsequent PARR analysis, literature has documented the feasibility of conducting this technique on frozen samples and cytological

preparations (Keller et al. 2016; Waugh et al. 2016; Ehrhart et al. 2019). Despite the potential reduction in the quality of preserved genetic material compared to fresh samples, this test requires DNA, which is considerably more stable than RNA. Therefore, using preserved DNA could be a viable alternative while awaiting a determination based on the accuracy of the cytological diagnosis. In figure 3, a decision tree is presented that addresses the minimally invasive techniques used in this study for the diagnosis of dogs suspected of multicentric lymphoma. This graphical representation suggests the flow of decisions and procedures adopted throughout the diagnostic process, providing a clear view of the steps involved in determining the final diagnosis.

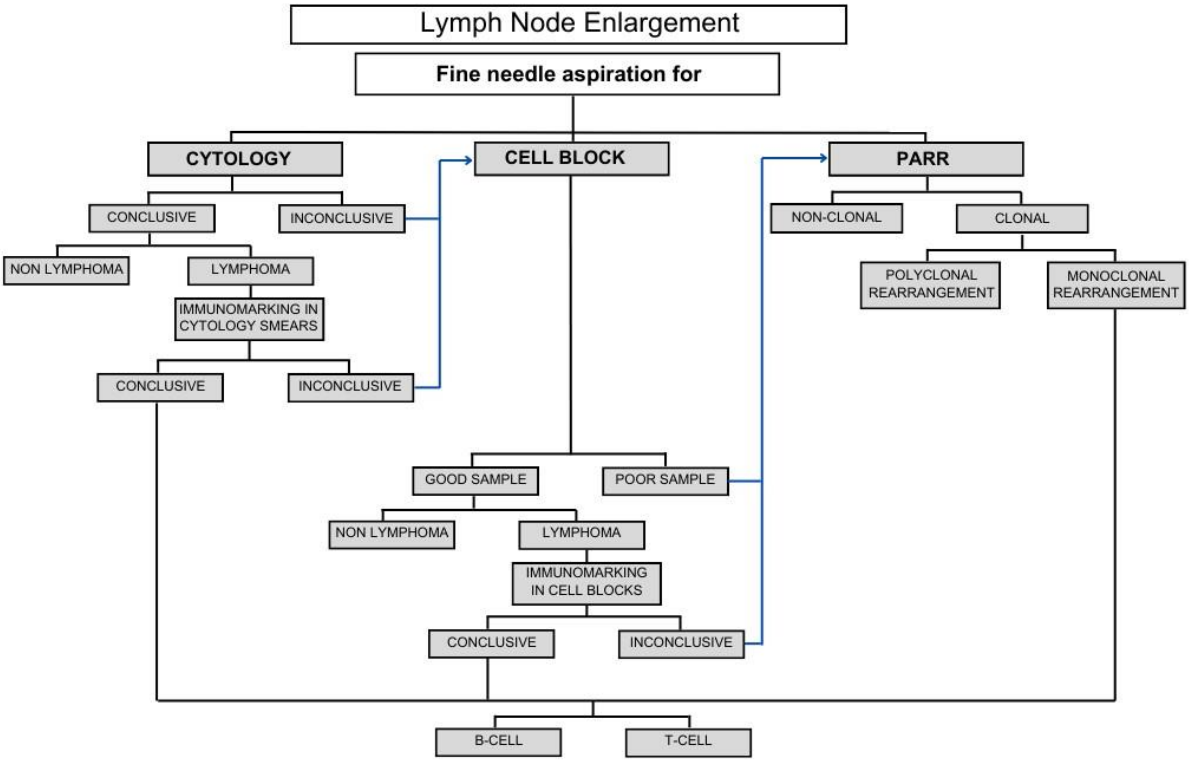

Figure 3. Decision tree proposal for the minimally invasive approach in the diagnosis of canine multicentric lymphoma

5. Conclusion

In summary, this study suggests the gains in cost and time saving without significant loss in accuracy through the use of minimally invasive approaches to the diagnosis and partial characterization of multicentric lymphoma in dogs, especially when it is urgent to start effective therapy for clinical signs regression. The authors' objective is not to discuss the importance of gold standard methods such as histopathology and immunohistochemistry of incisional lymph node biopsies, the only methods that make possible to classify lymphomas according to WHO

criteria. We believe that these methods should be performed whenever possible. The results obtained highlight the importance of a multidisciplinary approach in the diagnosis of this neoplasm and, as mentioned before, the authors believe to have supplied clinicians with information that will stimulate the acquisition of sufficient diagnostic material through minimally invasive techniques in the course of a single consultation.

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Conflicts of Interest

The authors declare no conflicts of interest.

Chapter III

Experimental Work

Multiple myeloma in dogs: Use of the cell block technique as a new diagnostic tool

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After being reviewed by the evaluation committee and including their suggestions.

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Maria Joana Dias: helped to conceptualize, clinical care, research, writing—review and editing.

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Jorge Correia: contributed to conceptualization, writing—review, editing and supervision.

Abstract

Background: The diagnosis of multiple myeloma (MM) in dogs may be challenging and complex. The cell blocks are a diagnostic technique that allows the characterization of neoplastic cells and, therefore, might help in the diagnosis of atypical MM.

Objective: The objective of the present work is to describe three clinical cases in which the cell blocks and immunohistochemistry contributed to the definitive diagnosis of canine MM.

Methods: Three dogs, one female, and two males, with different clinical signs, were presented for consultation with anemia, hyperproteinemia with monoclonal gammopathy, and the presence of plasmacytosis in the bone marrow. Cytologic analysis of the spleen was performed in two dogs and was suggestive of the presence of lymphocytes or plasma cells of a neoplastic nature in one of the cases and plasma cell hyperplasia associated with extramedullary hematopoiesis in the other. Given the hypotheses of lymphoid neoplasms with a plasma cell phenotype, cell blocks from fine needle aspiration were performed for immunohistochemical analysis with anti-CD3, CD20, CD79αcy, PAX5, and MUM1 antibodies.

Results: The results revealed positive staining for MUM1 in 80% of the cells in the spleen cell block and for CD20 and MUM1 in 70% of the cells in the bone marrow cell blocks, with negative staining for the other antibodies. The immunophenotyping results allowed the diagnosis of MM in the three cases and excluded other lymphoid neoplasms.

Conclusions: This work reinforces the importance of using cell blocks in the diagnosis of neoplasms by demonstrating their potential to aid the diagnosis of MM.

Keywords: Bone marrow, Canine, CD20, Diagnosis, MUM1.

1. Introduction

Multiple Myeloma (MM) is a clonal proliferation of malignant plasma cells with origin in the bone marrow and is associated with a monoclonal gammopathy and multiple osteolytic bone lesions. It is considered a primary bone tumor and is uncommon in dogs and cats, with no apparent sex or breed predisposition (Valli et al. 2016; Thompson and Dittmer 2017). It represents <1% of all malignant tumors in animals, accounting for about 8% of all canine hematopoietic tumors (Matus et al. 1986). The pathology associated with MM results from high levels of circulating myeloma proteins (M component) and the infiltration of organs and/or bones with neoplastic cells (Vail 2016; Vail et al. 2020). Pathologic conditions associated with this neoplasm include bone disease, hemorrhagic diathesis, hyperviscosity syndrome, kidney disease, hypercalcemia, immunodeficiency, cytopenias, and heart failure (Vail et al. 2020).

The diagnosis of MM is made through the association of several clinical abnormalities, mainly through the observation of plasmacytosis in the bone marrow, the presence of osteolytic bone lesions, and the demonstration of serum or urinary M proteins (monoclonal gammopathy or Bence-Jones proteinuria) (Valli et al. 2016; Vail 2016; Vail et al. 2020). A list of differential diagnoses should be considered, which include B-cell lymphomas and leukemia, extramedullary plasmacytoma, chronic infections (e.g. ehrlichiosis, leishmaniasis), and monoclonal gammopathy of unknown significance (Vail 2016; Vail et al. 2020). Definitive diagnosis of MM in dogs generally requires the evaluation of a bone marrow aspirate or core biopsy (Vail 2016; Vail et al. 2020), although other complementary tests may also be important to exclude differential diagnoses, such as immunohistochemistry, flow cytometry, and PCR for antigen receptor rearrangement (PARR) (Vail et al. 2020; Wachowiak et al. 2022).

The interest in minimally invasive techniques and tests that help cytology diagnoses has grown in the last few years (Melega et al. 2020). The cell block technique, which has been used for decades in human medicine (Shidham 2019), was recently adopted as a technique in veterinary medicine and applied to the diagnosis of cavitary effusions and neoplastic and infectious processes (Menezes et al. 2016; Heinrich et al. 2019; Marcos et al. 2019; Melega et al. 2020; Marrinhas et al. 2022). Several techniques can be used to make these blocks, all involving two distinct phases - the preparation, where there are variations, and the processing, which is similar in all methods (Melega et al. 2020). Although they can be used with various biological specimens, cell blocks have been more frequently recommended for cytologic samples that are concentrated and embedded in paraffin (Shidham 2019). One study validated the usefulness of HistoGel cell blocks in routine veterinary diagnostic cytology and confirmed that immunohistochemistry can be performed on HistoGel embedded cytologic samples without any interference using standardized methods developed for histopathology (Joiner and Spangler 2012).

Recently, some studies have used immunohistochemical techniques on cell blocks of cytologic samples in small animals with good results, turning this practice recognized as a complementary tool to one in which cytologic diagnoses can be made; this is especially true for neoplastic diseases. In fact, definitive diagnoses of nodal and gastrointestinal lymphomas, mesenchymal gastrointestinal tumors, mesotheliomas, and carcinomas have already been achieved (Taylor et al. 2013; Heinrich et al. 2019; Marcos et al. 2019; Marrinhas et al. 2022) but there are very few reports of its applicability in the diagnosis of canine MM (Marcos et al. 2018).

The objective of this work is to present three cases where the preparation of cell blocks using HistoGel is followed by immunohistochemical analysis for the diagnosis of MM in dogs.

2. Materials and methods

2.1 The cell block technique with HistoGel and immunophenotypic analysis

The cell blocks were performed from bone marrow, spleen, and liver aspiration. The aspirated samples were placed in Eppendorf tubes with 10% buffered formalin for a 24-hour fixation. The cell blocks were prepared with HistoGelTM (Thermo Fisher Scientific®) according to the manufacturer's recommendations. HistoGel is solid at room temperature and must be liquefied for use by heating to 50ºC in a water bath. After the first centrifugation of the fixated samples, the cell pellet was combined with 200-400µl of liquefied HistoGel. Then, the samples were mixed with the gel matrix and centrifuged again for cell concentration. The embedded samples were then chilled at 4°C for 3-5 min to allow complete solidifying. HistoGel-encapsulated samples were divided in half, transferred to a histology cassette, and taken to automated histologic processing. Cell block sections were stained with Hematoxylin and eosin (HE) to assess the quality of the material and the viability to proceed to cellular characterization by immunohistochemistry.

Immunostaining of cell block cells was performed using the EnVisionTM Kit (Dako, Agilent, Santa Clara, CA, USA) with the following antibodies: anti-CD3 (polyclonal antibody, Dako, 1:400 dilution) as a T lymphocyte marker, anti- CD20 (polyclonal antibody, Biocare Medical, Pacheco, CA, USA, 1:50), PAX5 (monoclonal antibody, clone SP34, Ventana, Tucson, AZ, USA, ready to use), and anti-CD79αcy (monoclonal antibody, clone HM57, Dako, 1:200), as B lymphocyte markers, and anti-MUM1 (monoclonal antibody, BC5 clone, Biocare Medical, 1:180 dilution) as a plasma cell marker.

2.2 Presentation of cases

A 13-year-old neutered male mongrel dog (Case #1), a 9-year-old neutered male Flanders Bouvier (Case #2), and a 9-year-old spayed female Australian Shepherd (Case #3) were presented for consultation. All animals had different clinical histories: lethargy, reduced physical activity, and lameness in case #1; anorexia, chronic diarrhea, episodes of vomiting and weight loss in case #2; epistaxis, acute diarrhea, pale mucous membranes, and body condition score 4 out of 9 in case #3.

CBC showed that the three cases had normochromic normocytic anemia (Hematocrit 31, 35.8, and 24.5%, respectively, reference interval [RI]: 37-55%). Cases #2 and #3 had thrombocytopenia (89000, 138000/µl, respectively, RI:200000-500000/µl), and case #2 also had leukopenia (3500/µl, RI:6000-17000/µl). All cases showed hyperproteinemia (9.04, 7.95, 8.36 g/dL, respectively, RI: 5.0-7.5 g/dL) with hyperglobulinemia (7.04, 5.93, 6, 0 g/dL, respectively, RI:2.8-4.0 g/dL) and normal serum ionized calcium values. Due to epistaxis, blood clotting tests were also performed in case #3, showing an increase in activated partial thromboplastin time (APTT) (108 seconds, RI:72-102 seconds).

After evaluating the analytical results, the proteinogram and screening of vector-borne diseases were carried out. Proteinograms revealed the presence of hyperproteinemia (9.8, 7.8, 9.5, respectively, RI: 5.5-7.5g/dL) associated with monoclonal gammopathy in the three cases (4.9, 3.4, 4.2, RI:0.3-1.02g/dL). All animals presented normal or low values for α-1 globulin (0.5, 0.3, 0.4, respectively, RI:0.32-0.75g/dL), α-2 globulin (1.1, 0.7, 1.1, respectively, RI:0.5-1.17g/dL), β globulin (0.5, 0.7, 0.6, respectively, RI:0.93-2.0g/dL) and a low A/G ratio (0.4, 0.51,0.51, respectively, RI:0.6-1.1).

All cases were negative for *Ehrlichia canis*, *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *Dirofilaria immitis* (SNAP-4Dx, IDEXX Laboratories), and *Leishmania* sp. (<1/20 for all - valuation criteria > 1/160, enzyme-linked immunosorbent assay). Cases #2 and #3 were also negative for *Babesia canis* (1/32 for both -valuation criteria >1/32, indirect immunofluorescence). Considering the presence of monoclonal gammopathy and the negative screening for infectious processes, a neoplastic origin was considered. Urine samples were collected from all cases for urinary Bence-Jones protein evaluation by immunofixation, the results of which were negative. Imaging studies were performed. Thorax radiographs were taken in the laterolateral and ventrodorsal positions. None of the cases showed signs of bone lysis in these radiographic projections, and case #2 presented hepatomegaly. In the abdominal ultrasound of the 3 dogs, only case #1 showed mild splenomegaly, and case #2 showed splenomegaly, hepatomegaly, and enlargement of hepatic and mesenteric lymph nodes.

Splenic ultrasound-guided aspiration punctures were performed in cases #1 and #2, and liver punctures in case #2. Cytologic examination of these samples revealed, in case #1, the presence of a neoplastic process involving the proliferation of round cells with morphology

compatible with lymphocytes or plasma cells of a neoplastic nature (Figure 5). In case #2, the cytologic diagnosis suggested the presence of metabolic overload with occasional plasma cells in the evaluation of liver smears and plasma cell hyperplasia associated with extramedullary erythropoiesis in the spleen.

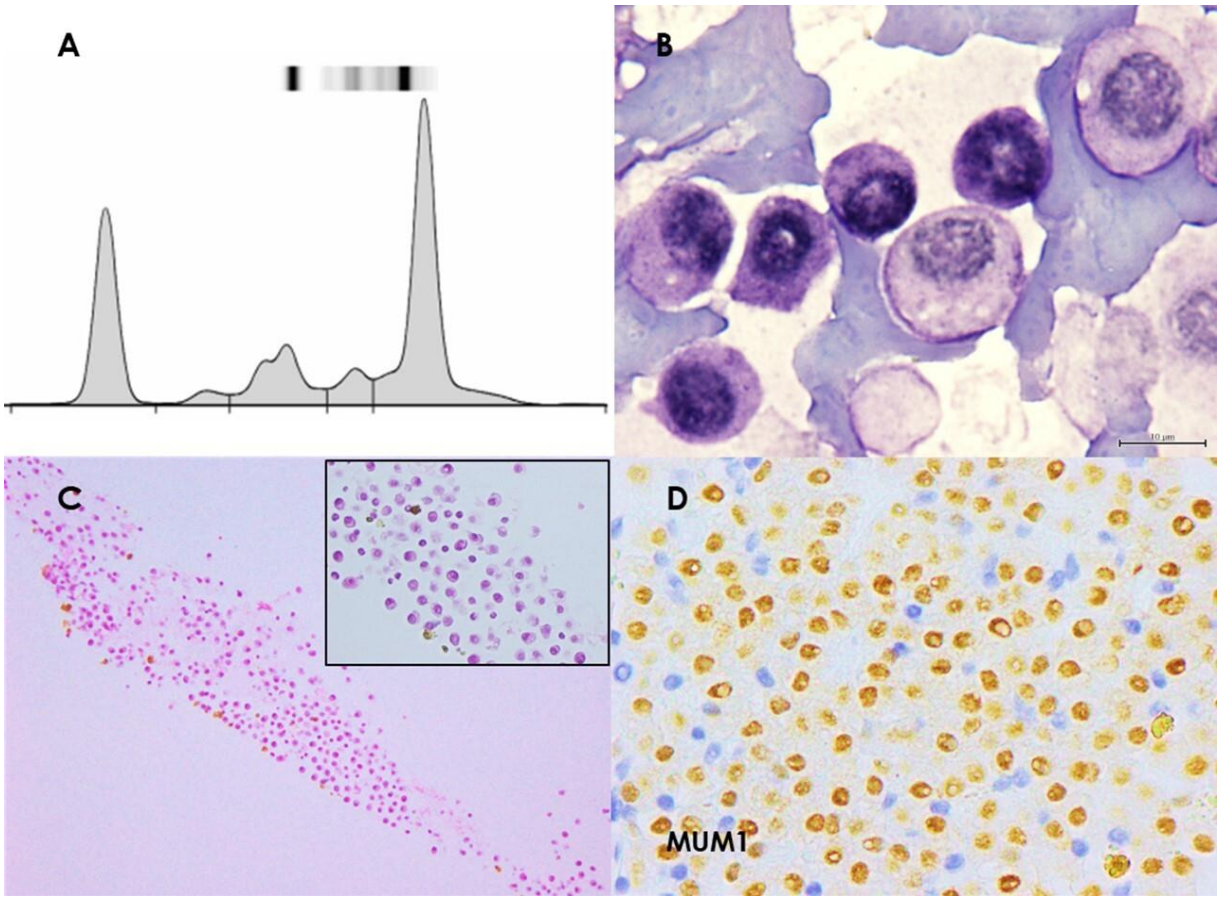

Figure 4. Multiple myeloma in a dog with spleen dissemination. Case #1 – (A) Serum protein electrophoresis with monoclonal peak in a 13-year-old dog with multiple myeloma (B) Microphotograph of the cytological examination showing infiltration of the spleen by round cells compatible with lymphocytes or plasma cells of a neoplastic nature (Giemsa, ×1000). (C) Microphotography of a section of cell block performed from splenic aspirate (H&E, ×40 and box ×400). (D) Microphotographs of the immunohistochemistry technique in cell block from spleen aspiration, positive staining, nuclear, strong intensity for anti-MUM1 antibody (Mayer's hematoxylin, ×400).

With the association of the results obtained and the exclusion of vector-borne diseases, bone marrow aspiration punctures were performed in all cases. The myelogram revealed a significant increase in plasmacytoid cells in all cases, presented at 30%, 16%, and 70%, respectively (RI: <5%). In cases #1 and #3, these cells also showed morphologic alterations, such as moderate anisocytosis and anisokaryosis, polarized nuclei, reduced chromatin density, evident nucleoli, and the presence of cytoplasmic vacuolation and binucleation. In

addition, polymerase chain reaction (PCR) was performed from bone marrow samples for vector-borne diseases, all with negative results.

Due to the suspicion of lymphoid neoplasms with a plasma cell phenotype, cell blocks of different cytologic samples and immunohistochemistry were performed. In the splenic cell block of case #1, immunohistochemistry was MUM1-positive, with strong nuclear staining in 80% of the cells present and CD20-negative (Figure 4). In cases #2 and #3, there was strong membrane marking for CD20 and nuclear marking for MUM1 in 70% of the cells present, with a large part overlapping for the two markers in the bone marrow cell blocks (Figure 5). In case #2, the evaluation of the liver showed that the organ did not seem to be affected by the neoplasm and the in spleen 80% of the neoplastic cells tested positive for both the CD20 and MUM1 markers, showing significant overlap between the two. However, the scarcity of cells in the cell block did not allow for a safe diagnosis. None of the cases showed significant immunoreactive staining for CD3, PAX5, and CD79αcy (staining ≤ 10%). These results observed in the characterization of the cell type by immunohistochemistry in the three cases reinforced the definitive diagnosis of multiple myeloma.

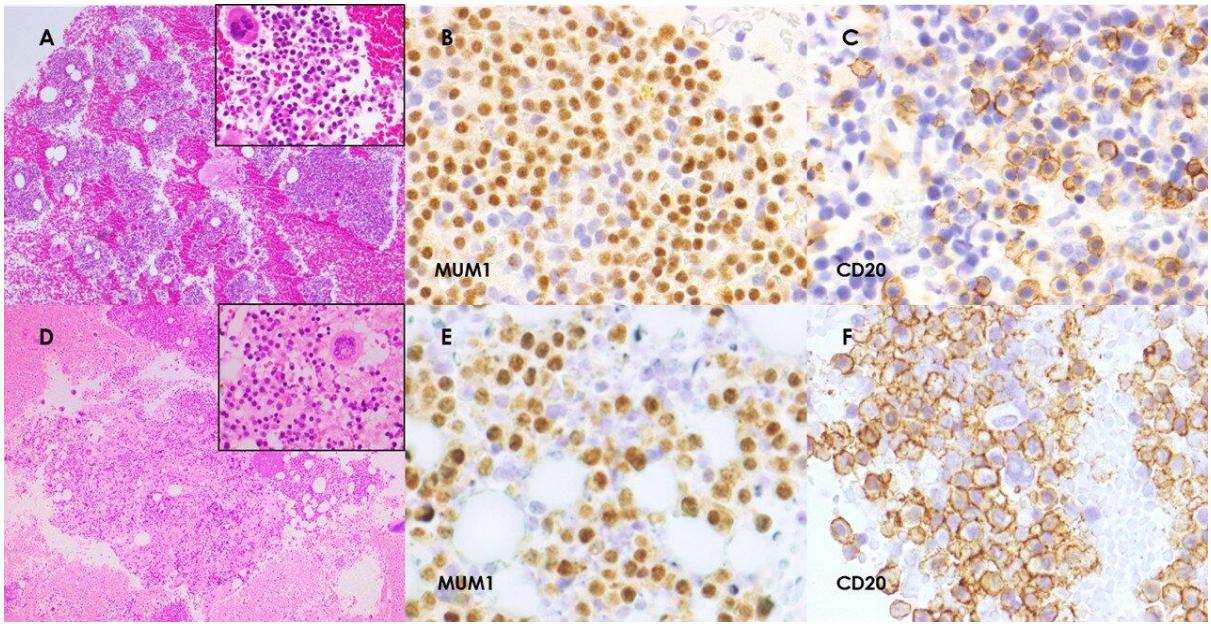

Figure 5. **Multiple myeloma in two dogs, the use of cell block and immunohistochemistry as a diagnostic tool. Case #2 (A, B, C) and case #3 (D, E, F). (A, D) Microphotography of a section of cell block performed from bone marrow aspirate (H&E, ×40 and box ×400). (B, C, E, F) Microphotographs of the immunohistochemistry technique in cell block from bone marrow puncture, (B, E) positive staining, nuclear, strong-intensity for anti-MUM1 antibody and (C, F) positive staining, membrane and strong-intensity for the anti-CD20 antibody (Mayer's hematoxylin, ×400).**

With the definitive diagnosis, the three animals were sent to the oncology service for proper treatment. Chemotherapy protocol included prednisone (0,5mg/Kg/SID) and melphalan (7 mg/m2/SID). Melphalan was administered in a pulsatile manner for five consecutive days with 16 days of rest. The animals survived chemotherapy treatment for two, 13, and 43 months, respectively. They all died.

3. Discussion

This study illustrates the complexity involved in defining a diagnosis of MM in dogs and highlights the importance of the use of Histogel cell blocks and immunohistochemistry of cell block sections in aiding the diagnosis of this rare neoplasm in three cases.

In the present cases, clinical signs, and analytical abnormalities, especially monoclonal gammopathy, were highly suggestive of infectious, neoplastic or idiopathic processes. After screening for vector-borne diseases and carrying out imaging tests and cytologic evaluations of organs and tissues, the hypotheses of hyperplastic or neoplastic processes of lymphoplasmocytic origin were considered.

Some infectious agents, such as *Ehrlichia* and *Leishmania*, can induce the production of clonal immunoglobulins. These can be differentiated from MM by identifying frequent plasma cells of abnormal morphology and through serologic or molecular tests for the diagnosis of the agents in question (Valli et al. 2016; Thompson and Dittmer 2017). Malignant plasma cells normally produce an overabundance of a single type or component of immunoglobulin (Vail et al. 2020). In humans, it is found in the serum or urine of 97% of patients with myeloma, except those classified as non-secretors (Kyle et al. 2003). Although the three cases reported were negative for the M component in the urine, all showed hyperglobulinemia with non-infectious serum monoclonal gammopathy, which supported the suspicion of neoplasia.

The presence of osteolytic lesions is a strong indication for the diagnosis of MM in dogs, but they may not be present (Thompson and Dittmer 2017). In the absence of these lesions, the diagnosis of canine MM can also be made by associating medullary plasmacytosis with a progressive increase in M proteins or with the identification of plasma cell clonality (Vail et al. 2020). In the three cases, in the absence of osteolytic lesions, immunostaining of bone marrow and/or spleen cell blocks reinforced the diagnosis of MM and showed splenic dissemination in cases #1 and #2. Despite the small number of cases described using these minimally invasive techniques in the diagnosis of MM in dogs, their use should be considered important in cases with unusual presentations, whereas in more typical cases, they can help to reinforce the diagnosis more objectively.

A study in human patients suggests that splenic aspirate cell blocks can be used as an alternative to splenectomy or biopsy in the diagnosis of lymphoproliferative disorders involving the spleen and that immunohistochemistry can be useful to confirm or to exclude malignancy

processes, such as lymphomas (Ramdall et al. 2006). In the present work, the presentation of relevant results in the characterization of the cell type involved and in the identification of a neoplastic process supports the use of cell blocks with immunohistochemistry from splenic aspirates in dogs with MM and splenomegaly to complement the cytologic diagnosis and highlight splenic dissemination. Organomegaly and extramedullary plasmacytosis, despite being present in two of the reported cases, appear to be more common in cats than in dogs with MM, with the liver and spleen being the most affected organs (Patel et al. 2005).

Definitive diagnosis of canine MM usually requires performing a bone marrow aspiration or core biopsy (Vail 2016; Vail et al. 2020). In an evaluation of histopathologic parameters, cell blocks made from bone marrow aspirates showed similar results to biopsy fragments, suggesting that they can be used as an alternative for the evaluation of diseases that affect this tissue (Varjão et al. 2021). Currently, recommendations require more than 20% of plasma-cells present in the cytologic examination of the medullary aspirate in the presence of this neoplasm, with counts up to 5% considered normal for the species (Thompson and Dittmer 2017; Vail et al. 2020). The immunohistochemical technique in cell blocks of bone marrow aspirates has already been considered a useful tool in the parasitologic diagnosis of canine visceral leishmaniosis and the detection of micrometastasis of carcinoma in bone marrow in dogs and cats (Taylor et al. 2013; Menezes et al. 2016). In the present work, the identification of approximately 70% of plasma cells in the immunohistochemistry of medullary cell blocks from cases #2 and #3 revealed and confirmed the potential of this complementary technique for the definitive diagnosis of MM in dogs, mainly in the case #2, which had presented only 16% of plasmacytoid cells in the cytologic examination of the bone marrow aspirate.

Cell blocks have stood out in the diagnosis of neoplastic processes, obtaining good results mainly from the characterization and immunophenotyping of the cells involved (Fernandes et al. 2016; Menezes et al. 2016; Heinrich et al. 2019; Marcos R et al. 2019; Melega et al. 2020; Marrinhas et al. 2022). When performed from organ and tissue aspirates, the cell block technique is considered an excellent alternative to cytologic immunostaining when incisional biopsies are not possible due to the fact that the comparison between antibody staining is very precise since the same cells are being evaluated, regardless of the number of antibodies used (Peleteiro 2011). Although fine needle aspiration cytologic examination is a quick procedure, a low-cost tool with high sensitivity and specificity in the diagnosis of lymphoproliferative processes, it has its limitations, and other techniques are being applied to improve its diagnosis (Talheim et al. 2013; Fernandes et al. 2016; Heinrich et al. 2019). In humans, cell blocks have already been used for the diagnosis of pleural effusions associated with MM (Chen et al. 2018), and, to the best of our knowledge, the present work describes, for

the second time but with a slightly greater number of cases, the use of this technique to aid in the diagnosis of rare MM cases in Veterinary Medicine.

Although immunohistochemistry is mostly applied in the diagnosis of other neoplastic processes of plasmacytic origin, it can also be useful in MM (Vail et al. 2020; Wachowiak et al. 2022). With the evaluation of the results obtained, neoplastic processes involving plasma cells or lymphocytes were strongly considered, and therefore, immunohistochemistry in cell blocks was performed to differentiate the cell type involved. In the present study, the three dogs showed positive immunohistochemical staining for MUM1, with CD20 co-expression in two cases, and were negative for the other antibodies, which confirmed the presence of plasma cells in the cell blocks performed and supported the diagnosis of MM. Multiple myeloma oncogene 1 (MUM1) is involved in the differentiation of lymphoid cells, particularly in the production of plasma cells (Ramos-Vara et al. 2007). Immunostaining for MUM1 is expected in terminal plasma cell differentiation concomitant with PAX-5 downregulation (Willis et al. 2014). The absence of staining for PAX5, CD79a excluded the presence of B lymphocytes and CD3 of T lymphocytes, although expression of MUM1 associated with CD3 in peripheral T-cell lymphoma or CD20 in diffuse large B-cell lymphoma has recently been reported in dogs (Riccardi et al. 2023). Although CD20 is a transmembrane phosphoprotein commonly expressed at different stages of B lymphocyte differentiation, its atypical expression in plasmacytic neoplasms is poorly described, and it has already occurred in cases of MM in humans and extramedullary plasmacytomas in dogs (Ramos-Vara et al. 2007; Jian et al. 2022).

The presence of hypercalcemia, Bence-Jones proteinuria, and extensive bone lysis are considered negative prognostic factors in the dog (Vail et al. 2020), but none of these alterations were observed in the present case reports. However, the presence of CD20 marking in neoplastic plasma cells from the cell blocks of two animals may be related to longer survival times after diagnosis and initiation of adequate treatment. A study of a specific cytogenetic group of humans newly diagnosed with MM proposes that unusual CD20 expression may be a favorable prognostic factor with a tendency towards longer survival, (Jian et al. 2022) which, despite the small number of cases showed in this study, suggests that the use of cell block and immunohistochemistry may also be relevant in the investigation of a possible prognostic factor in dogs with MM, which should be studied.

4. Conclusions

This study presents the use of the association of cell blocks and immunohistochemistry for the definitive diagnosis of MM in dogs, reinforces the importance of using these minimally invasive techniques in the diagnosis and cellular characterization of neoplasms in Veterinary Medicine, and exposes an unusual CD20 expression in MM, which should be considered in

future investigations into its causes, clinical significance and prognostic factor in the canine species.

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Conflict of Interest: The authors declare no conflict of interest.

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Chapter IV

Experimental Work

Co-Expression of T- and B-Cell Markers in a Canine Intestinal Lymphoma: A Case Report

Pâmela Cristina Lopes Gurgel Valente, Maria Conceição Peleteiro, Sandra Carvalho, Rodolfo Oliveira Leal, Constança Pomba, António Duarte and Jorge Correia. Animals. 2022 Dec 14;12(24):3531.

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Author Contributions:

Pâmela Cristina Lopes Gurgel Valente: Conceptualization, laboratory processing, data analysis, research, writing—original draft preparation, writing—review, editing and funding acquisition.

Maria Conceição Peleteiro: helped to conceptualize, laboratory processing, research, writing—original draft preparation, writing—review, editing and supervision.

Sandra Carvalho: contributed to the laboratory processing.

Rodolfo Oliveira Leal: helped to conceptualize, clinical care, research, writing—review and editing.

Constança Pomba: contributed to the supervision.

António Duarte: contributed to the supervision.

Jorge Correia: contributed to conceptualization, research, writing—review, editing and supervision.

Simple Summary:

The diagnosis of chronic gastroenteropathy in dogs can be complex, highlighting the relevance of histopathology allied to immunohistochemistry in the identification of the involved cell types, contributing to the exclusion of differential diagnoses, including special types of intestinal lymphomas. However, rare cases of the co-expression of some immunomarkers in lymphoid cells can make the interpretation of results particularly difficult. This report presents a case of a dog with a diagnosis of intestinal lymphoma, in which the co-expression of CD3 and CD20 in lymphoid cells was identified in immunocytochemistry and immunohistochemistry, making it necessary to identify lymphoid clonality using polymerase chain reaction for antigen receptor rearrangement (PARR) for a precise diagnosis**.**

Abstract:

An 8-year-old female neutered Labrador retriever was presented for a second opinion consultation due to vomiting and lethargy, having failed to respond to symptomatic therapy. Blood analysis revealed hyperbilirubinemia and hypoalbuminemia, associated with hypocobalaminemia. An abdominal ultrasound identified diffused bowel thickening and hypoechoic hepatomegaly. An ultrasound-guided liver fine-needle aspiration was performed for cytology and also for cell block immunocytochemistry. Gastric and duodenal biopsies were collected by gastroduodenoscopy. Liver cytology showed numerous lymphocytes, suggesting lymphoma at the hepatic infiltration stage, and immunocytochemistry in the cell block of the hepatic aspirate indicated co-expression of CD3 and CD20 in the lymphoid cells present. The histopathology of gastric and duodenal biopsies supported the hypothesis of gastrointestinal lymphoma due to heavy lymphoid infiltration of the gastric epithelium and intestinal mucosa, including the villi. Concurrent immunohistochemistry was performed using CD3, CD20, PAX5, and CD79αcy antibodies. Immunostaining was positive for CD3 and CD20, which overlapped populations of lymphoid cells, and was negative for all other antibodies. In the clonality test, was confirmed the monoclonal rearrangement of T-cell gamma receptors. The final diagnosis was type 2 enteropathy-associated T-cell lymphoma with hepatic infiltration. Co-expression was examined in conjunction with the PARR result in the presence of T-cell monoclonal rearrangement.

Keywords: Dog; Intestinal lymphoma; Enteropathy; Co-expression; CD3; CD20; Clonality

1. Introduction

Primary gastrointestinal tract lymphoma, although less frequent in dogs than in cats, is cited as one of the main neoplastic causes of chronic gastroenteropathy in domestic animals (Vail et al. 2013; Munday et al. 2016). It is most commonly reported in the small intestine, stomach, and colon. Several segments of the intestine may be involved, and there is often a dispersal to regional lymph nodes and the liver (Coyle and Steinberg 2004; Gieger 2011; Uzal et al. 2016).

Currently, it is known that this neoplasm originates mainly from T-lymphocytes in dogs and cats (Coyle and Steinberg 2004; Vail et al. 2013; Munday et al. 2016). When lesions are not transmural or histopathology is based on endoscopic biopsies, the diagnosis is considered a challenge, requiring more than the conventional histopathological evaluation to differentiate it from inflammatory bowel disease (IBD) (Munday et al. 2016).

In humans, the histopathological classification of the World Health Organization subdivides enteropathies associated with intestinal T-cell lymphoma (EATL) into types 1 and 2. This classification is also used in cats and, more recently, in dogs (Carrasco et al. 2015). Type 1 EALT is composed of large cells associated with necrosis and an inflammatory background, whereas type 2 is monomorphic median to small-sized cells with no inflammatory background and rarely with necrosis (Chandesris et al. 2010; Ferreri et al. 2011; Carrasco et al. 2015; Munday et al. 2016). Based on this classification, the most common primary intestinal lymphoma in cats is T-cell-associated type 2 enteropathy with marked epitheliotropism, which may be morphologically indistinguishable from IBD (Gieger 2011; Carrasco et al. 2015; Munday et al. 2016). In dogs, a higher frequency of cases associated with type 1 enteropathy is described, with or without accompanying inflammation and/or epitheliotropism, which makes these parameters much less important in diagnosis for this species (Gieger 2011; Carrasco et al. 2015; Munday et al. 2016).

With the combination of histopathological and immunohistochemical evaluations, the ability to differentiate intestinal lymphomas from IBD has improved. With these methods, it is expected that most neoplastic cells from intestinal lymphoid tumors will express either one of the T- or B-cell markers, but not both. However, there are already some reports of co-expression of CD3 and CD20 in canine lymphomas, including three cases of intestinal T-cell lymphomas associated with type 1 EATL (Brachelente et al. 2016; Noland and Kiupel 2018; Nicoletti et al. 2020). These findings reinforce the importance of PCR for antigen receptor rearrangement (PARR) for a definitive diagnosis of lymphoid cells in intestinal neoplasms (Vail et al. 2013, Munday et al. 2016; Carrasco et al. 2015; Noland and Kiupel 2018).

The objective of this work is to present the case of a dog with intestinal lymphoma with co-expression of CD3 and CD20, compatible with EATL type 2, that was also showing hepatic

infiltration.

2. Case Presentation

A neutered 8-year-old female Labrador retriever was presented to the Veterinary Teaching Hospital (HEV), Faculty of Veterinary Medicine, University of Lisbon for a second opinion consultation due to lethargy, anorexia, and vomiting for 10 days, refractory to symptomatic treatment. Blood tests had been previously performed by the referring veterinarian, showing a normal complete blood count, increased alanine aminotransferase (ALT) 185 IU/L (RI: 10–78 IU/L), and alkaline phosphatase (ALP) 188 UI/L (RI: 7–83 UI/L), total protein 4.9 g/dL (RI: 5.4–7.8 g/dL), and hypoalbuminemia (albumin 1.7 g/dL, RI: 2.2–3.5 g/dL). The animal was hospitalized for symptomatic treatment with intravenous fluids and symptomatic treatment including metronidazole (10 mg/Kg every 12 h), amoxicillin with clavulanate (20 mg/Kg every 12 h), omeprazole (1 mg/Kg every 12 h), maropitant (1 mg/Kg every 24 h), and dexametasone (0.2 mg/Kg every 24 h).

On physical examination, the dog was prostrated, with pink mucous membranes, normal abdominal palpation, no peripheral lymph node enlargement, although with presence of hematochezia. The capillary refill time (CRT) was 2 s, the heart rate was 168 bpm, the respiratory rate was 16 cpm, the pulse was strong, and the temperature was 39.2 ◦C. At this point, the differential diagnosis for acute vomiting was established, detailing a digestive cause (infectious gastroenteritis, protein-losing enteropathy, primary vs. secondary lymphangiectasia caused by inflammatory bowel disease or neoplasia). An extra-digestive origin, such as liver disease, pancreatitis, or protein-losing nephropathy, was not excluded at this stage. Taking into account increased liver enzymes, primary (toxic, infectious, or copper-associated hepatopathy or neoplasia) or secondary (reactional hepatopathy secondary to gastroenteropathy, for instance) hepatopathies were considered.

The animal was admitted for medical investigation, and blood and urine samples were collected for a new biochemistry assessment and urine analysis. Results showed hypoalbuminemia (1.49 g/dL, RI: 2.2/3.5 g/dL), hyperbilirubinemia (1.17 mg/dL, RI: 0.0/0.41 mg/dL), hypocobalaminemia (172 ng/L, RI: 275–590 ng/L), and increased canine-specific pancreatic lipase (cPLI), 418 µg/L (RI: 201–399 µg/L). Apart from bilirubinuria, urine analysis was unremarkable, and the UPC (Urine Protein/Creatinine ratio) was negative (0.18).

After evaluating the new results, a search for vector-borne diseases was also carried out (leishmaniosis, dirofilariosis, borreliosis, anaplasmosis, and ehrlichiosis) and found to be consistently negative.

An abdominal ultrasound exam was performed, which identified hypoechoic hepatomegaly with mild thickening of the gallbladder wall, stomach distention with marked hypomotility, and diffuse thickening of the entire intestine associated with small striations in the

duodenum and jejunum, with maintenance of layering. Hypoechogenic mesenteric lymph nodes were at the upper limit in terms of size, and the kidneys showed small calcifications. Mucosal striations associated with hypoproteinemia and hypoalbuminemia supported a possible protein-losing gastrointestinal disease. At this point, taking into account the concurrent context of hypoalbuminemia, the main differentials were a protein-losing enteropathy secondary to intestinal inflammation versus infiltrative neoplastic disease (involving the gastrointestinal and hepatobiliary tracts).

Given the suspicion of liver disease associated with an exudative enteropathy (primary, secondary, inflammatory, or neoplastic disease), an ultrasound-guided liver FNA was performed for a cytological examination and for cell block preparation with HistogelTM (Thermo Fisher Scientific®) for immunocytochemistry.

The liver cytological analysis showed numerous plaques of generally normal-sized hepatocytes, among which numerous lymphoid cells were observed, corresponding to a homogeneous population of small-to-medium-sized cells with a slightly indented nucleus of homogenous chromatin and no clearly evident nucleoli. Neutrophils were also observed, albeit in reduced numbers, together with rare macrophages with phagocytosed material in their cytoplasm. The cytologic diagnosis suggested an infiltrative lymphocytic proliferation.

Due to the presence of hypoalbuminemia, hypocobalaminemia, and suspected protein-losing enteropathy, a gastroduodenoscopy and colonoscopy (for ileal sampling) were suggested, but, due to the non-neglectable prolonged anesthetic risk, only the gastroduodenoscopy was performed. This exam revealed signs of erosive gastritis. The duodenal mucosa was irregular and particularly friable, showing diffusely increased granularity. Multiple duodenal and gastric biopsies were performed.

The histopathological analysis of the biopsies revealed, in the pyloric area of the stomach, marked surface irregularities with anfractuous crypts. The lamina propria showed moderate fibrosis with a decrease in the number of glands and mild inflammatory infiltration, mostly by mononuclear cells. Small, round lymphoid cells with clear cytoplasm were seen intensely infiltrating the lining epithelium and that of the crypts (Figure 6 A,B). No microbial agents were identified. In the duodenum, the mucosa showed marked infiltration of the lamina propria by the same type of small lymphoid cells with clear cytoplasm, more intense in the villi, which were shortened and fused. These small lymphoid cells were also heavily present in the villi epithelium (Figure 7 A, B), similarly to what was seen in the pyloric epithelium.

The histopathological diagnosis was chronic fibrous gastritis with hyperplasia of the epithelium in the pyloric antrum and severe epithelial infiltration by small lymphoid cells as well as neoplastic infiltration of the duodenum by the same type of small lymphoid cells with marked epithelial tropism in the villar, consistent with intestinal lymphoma.

Immunophenotyping of lymphoid cells in the biopsies and in the hepatic cell block prepared from the liver aspirate was performed using the EnVisionTM Kit (Dako, Agilent, Santa Clara, CA, USA) protocol with the following antibodies: anti-CD3 (polyclonal antibody, Dako, dilution 1:400) as a T-cell marker and anti-CD20 (polyclonal antibody, Biocare Medical, Pacheco, CA, USA, 1:50), PAX5 (monoclonal antibody, SP34 clone, Ventana, Tucson, AZ, USA, ready-to-use), and anti-CD79αcy (monoclonal antibody, HM57 clone, Dako, 1:200) as B-cell markers.

Antigen retrieval was performed at a PTLink station (Dako). Antigen retrieval was achieved with concentrated Tris/EDTA pH 9.0 for all antibodies except PAX5, for which retrieval was achieved with citrate buffer pH 6.0. Mayer's hematoxylin was used for background staining.

Immunohistochemistry showed strong CD3-positive cytoplasmic and membrane marking in 80% of the small lymphoid cells in both stomach and duodenal mucosae (Figures 6C and 7C). Additionally, there was also strong cytoplasmic and membrane marking for CD20 (Figures 6D and 7D) in a slightly smaller percentage of the lymphoid cells, estimated at 70%, with a clear overlap with the CD3 marking. There was no immunoreactive labelling for PAX5 and CD79αcy. The cell block prepared from the hepatic aspirate also revealed the co-expression of CD3 and CD20 in the lymphoid cells present.

Subsequently, the polymerase chain reaction for antigen receptor rearrangement (PARR) technique was performed using DNA extracted from paraffin blocks of the stomach and duodenum for B- and T-cells. The analysis revealed the presence of monoclonal rearrangement of the T-cell gamma receptor gene and did not amplify for B-cell receptors (Waugh et al. 2016) (Figure 8).

A definitive diagnosis of intestinal T-cell lymphoma with concomitant expression of B-cell markers and hepatic infiltration was finally issued. The prognosis was considered reserved.

Due to the intense weakness and the unfavorable prognosis, euthanasia of the dog was requested by the owners and necropsy was not authorized.

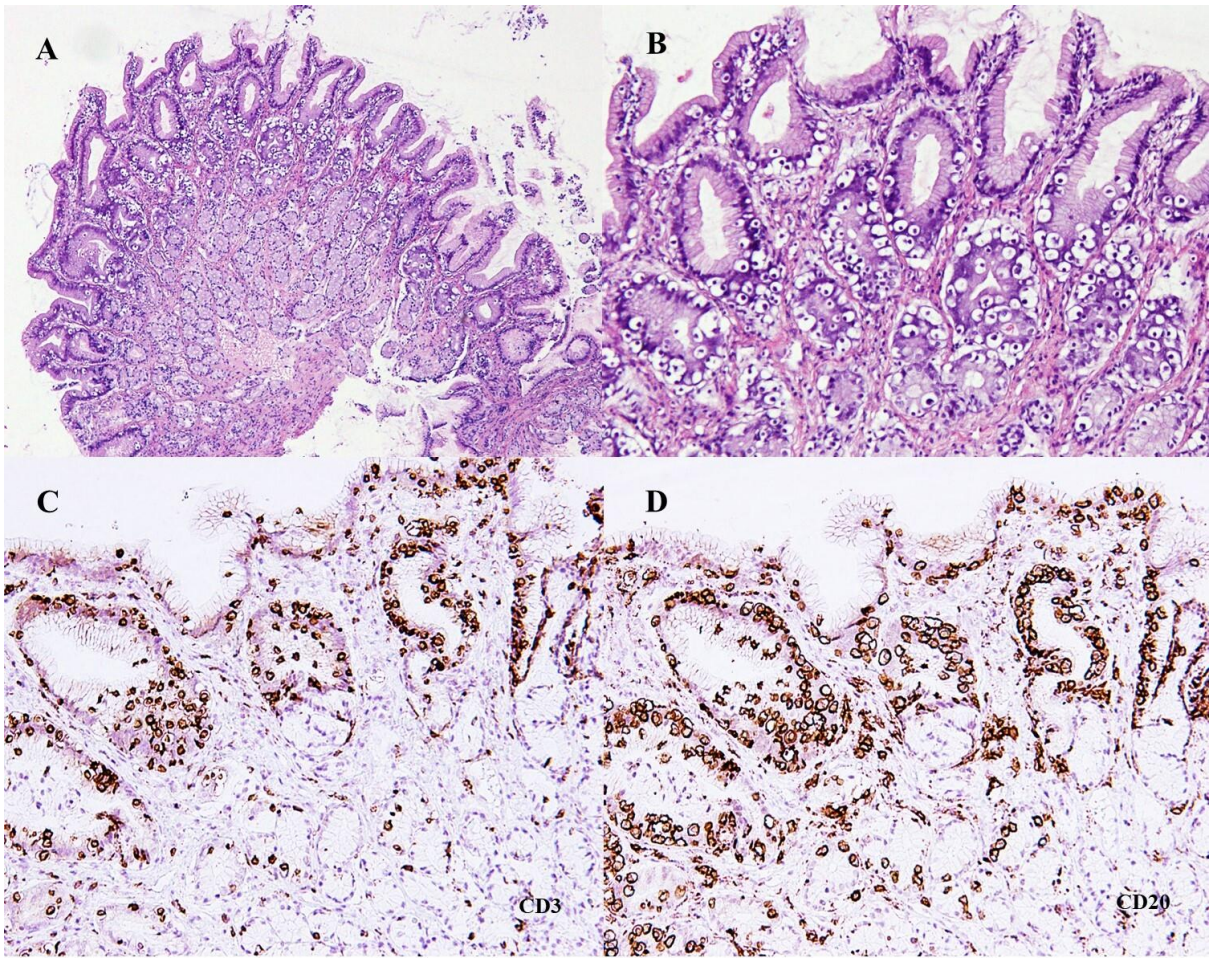

Figure 6. (A, B) Microphotographs of endoscopic biopsies of gastric pyloric mucosa showing infiltration of the superficial epithelium and one of the crypts by small lymphocytes (H&E, (A) ×40 and (B) ×100). (C, D) Immunohistochemistry for lymphoid T-cells (C) (anti-CD3) and B-cells (D) (anti-CD20) (Mayer's hematoxylin, ×100).

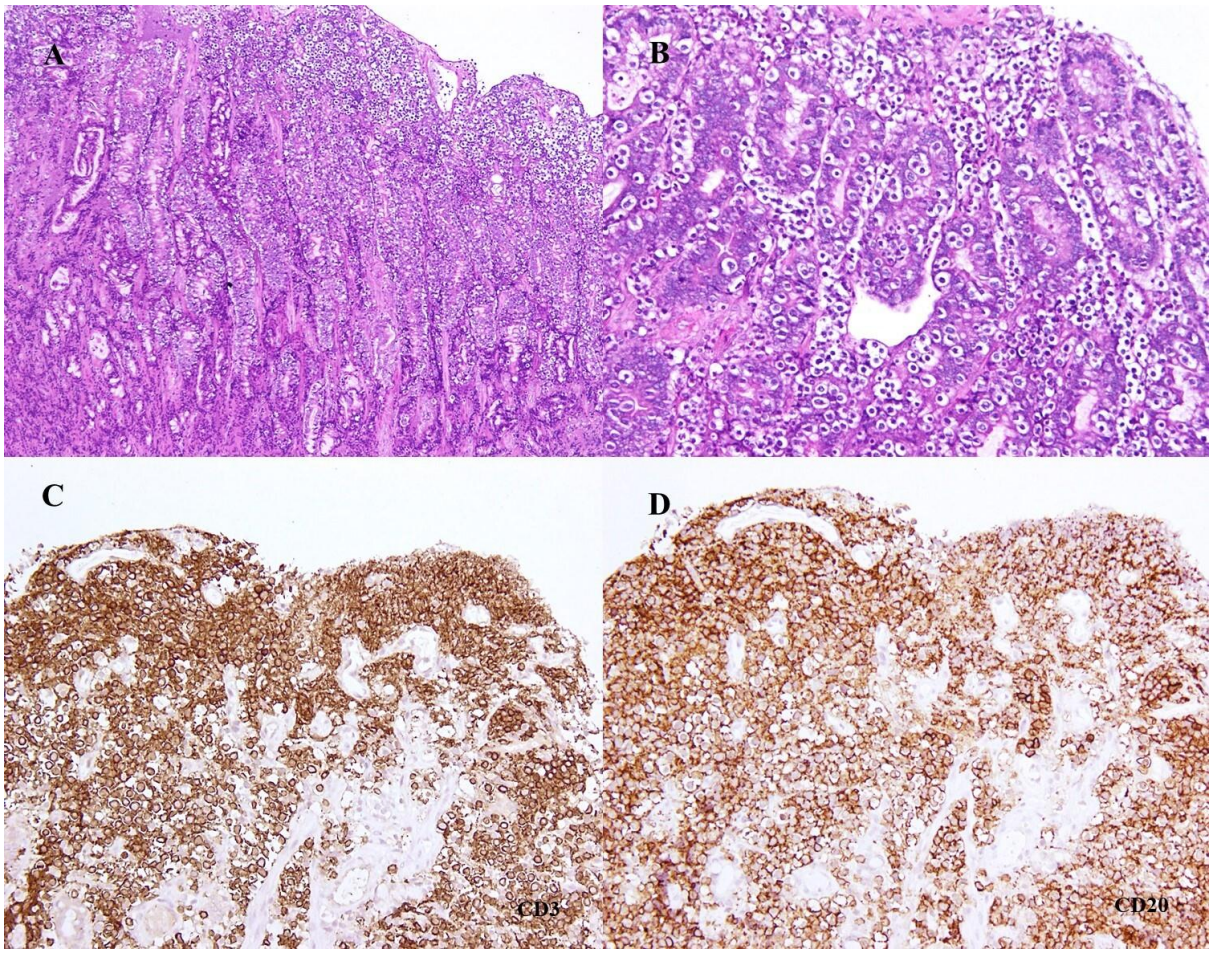

Figure 7. (A, B) Microphotographs of endoscopic biopsies of duodenal mucosa showing severe infiltration of the epithelium of the villi by small lymphoid cells identical to the ones in the gastric mucosa (H&E, (A) ×40 and (B) ×100). (C, D) Immunohistochemistry for lymphoid T-cells (C) (anti-CD3) and B-cells (D) (anti-CD20) (Mayer's hematoxylin, ×40).

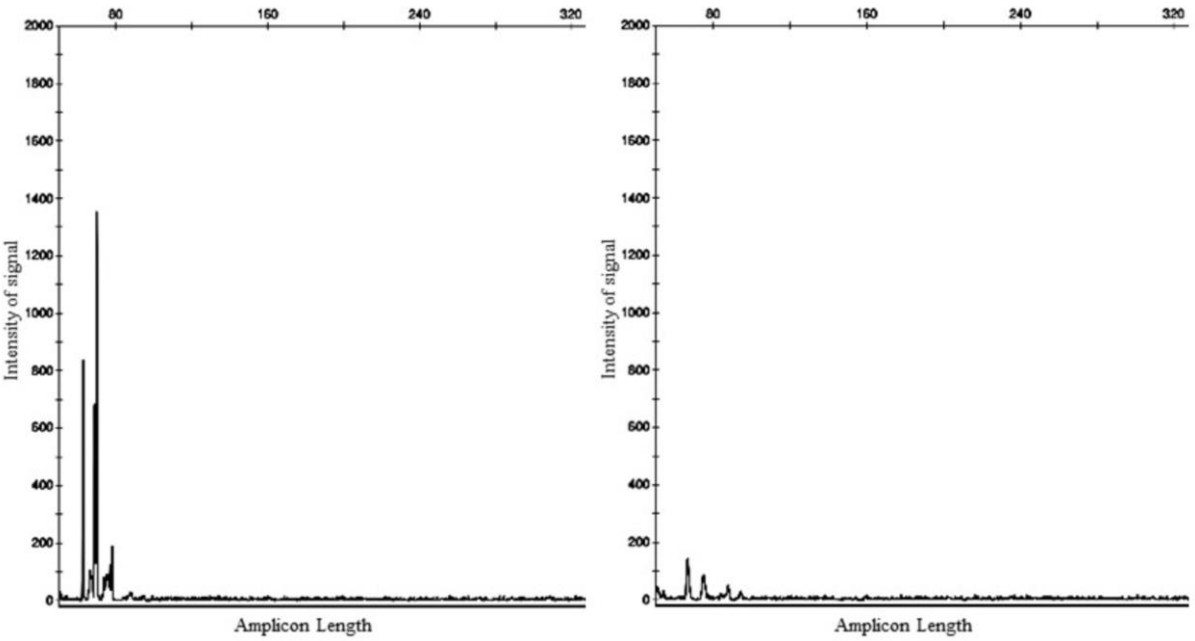

Figure 8. Molecular clonality analysis performed on DNA extracted from paraffin blocks using capillary electrophoresis in the 3500 Genetic Analyzer (Applied Biosystems®). Results show clonal amplification with a 55–82 bp peak of the T-cell receptor (TCRγ) on the lower left and negative or poor amplification for the B-cell receptor (IgH) on the lower right after using the PARR technique protocol. The x-axis is the length of the amplicon, and the y-axis is the intensity of the signal.

3. Discussion

This case report is a good example of the complexity of the diagnosis of chronic gastroenteropathy in dogs, highlighting the importance of employing the use of histopathology with immunohistochemistry and clonality assays in the identification of the cell types involved, contributing to a definitive diagnosis.

In the present case, the clinical signs, the analytic abnormalities, the poor response to therapy, and the ultrasound changes in the stomach, intestine, and liver were highly suggestive of severe enteropathy associated with reactive and/or neoplastic liver disease.

Hypoalbuminemia and hypoproteinemia can occur in cases of intestinal lymphomas in dogs (Gieger 2011; Frank et al. 2007; Sogame et al. 2018). The presence of hyperbilirubinemia and bilirubinuria without anemia, associated with a concurrent increase in liver enzymes, supported a hepatic cause, in this case, a consequence of the infiltrative neoplastic disease in the liver parenchyma (Stockham and Scott 2011).

In view of the suspicion of liver disease associated with enteropathy, and recognizing the potential limitations of this exam, a liver FNA was prioritized due to the poor health condition of the animal. The diagnosis of liver cytology was essential to address the possibility of gastrointestinal lymphoma, mainly because this organ is frequently affected in this neoplasm,

and cytology may be useful and quite sensitive for the diagnosis of round-cell hepatic tumors (Coyle and Steinberg 2004; Gieger 2011; Uzal et al. 2016). On the basis of liver cytology, the diagnosis of lymphoma is usually straightforward when it involves large cells. However, the presence of a small to-intermediate-sized lymphocyte population made it necessary to carry out additional diagnostic tests, mainly to rule out possible differential diagnoses such as lymphocytic hepatitis (Uzal et al. 2016; Siegel and Wiseman 2020).

Concerning the GI tract, the hypothesis of intestinal lymphoma was confirmed by histological analysis of the endoscopic biopsies of the stomach and intestine. In fact, the heavy lymphoid infiltration in the intestine was consistent with what is generally described for intestinal lymphoma, although a differential diagnosis with lymphoplasmocytic enteritis is always considered (Munday et al. 2016).

It is generally accepted that histopathological evaluation of incisional biopsies has greater value compared to endoscopic biopsies in the diagnosis of enteropathies, considering that all layers of the gastrointestinal wall can be evaluated (Gieger 2011), facilitating the differential diagnosis of IBD versus enteropathy-associated T-cell lymphoma type 2 (Carrasco et al. 2015).

However, the use of endoscopic biopsies is commonly accepted in clinical practice as less invasive and is especially recommended in debilitated animals. In the present case, the histopathological examination of endoscopic gastric and intestinal biopsies was a step further in the confirmation of the diagnosis of gastrointestinal lymphoma, with some uncommon findings, such as the marked presence of lymphoid cells in the gastric epithelium without infiltration of the mucosal lamina propria. In fact, the severe infiltration of the intestinal mucosa with a monomorphic lymphocyte population was also present in large numbers in the villous epithelium, obliterating the lamina propria:epithelial boundary, clearly suggesting lymphoma (Uzal et al. 2016). Considering the small size of the lymphoid cells and the absence of necrosis and an inflammatory background, the diagnosis of EALT type 2 was appropriate.

Immunohistochemistry is usually quite helpful in the differential diagnosis of IBD and intestinal lymphoma, as in IBD, positive labelling should be mixed between T- and B-cells (Carrasco et al. 2015). Therefore, it was surprising to find the labelling of the lymphoid cells with both T- and B-cell markers, with the same occurring in the cell block that was prepared from the cells obtained in the liver puncture. Cell blocks, prepared from aspirates of organs and tissues, are considered an excellent minimally invasive technique for the characterization and immunophenotyping of canine lymphomas (Fernandes et al. 2016; Heinrich et al. 2019). They are also an excellent alternative to cytology immunostaining when incisional biopsies are not possible as the comparison between antibody labelling can be accurate: the exact same cells are being evaluated regardless of the number of antibodies used (Peleteiro 2011).

Co-expression of CD3 and CD20 confirmed by immunohistochemistry is rare and has been described in only a few cases of canine T-cell lymphomas, such as cutaneous, intestinal, nodal, and peripheral lymphomas with infiltration of the heart and peripheral nerves (Brachelente et al. 2016; Nakagun et al 2018; Noland and Kiupel 2018; Nicoletti et al. 2020). Double lymphoid labelling has also been described in a cat, but in a B-cell lymphoma with expression of CD3, identified by clonality tests (Granum et al. 2015). Despite the co-expression of CD3 and CD20 observed in the present work, it is important to emphasize that a panel of three antibodies for B-cells was used, but we only anti-CD20 labelled the lymphoid cells. In fact, PAX 5 and CD79αcy are specific for particular stages of B-cell differentiation, but a good degree of overlapping is expected, which was not the case. The need to clarify the exact lineage of the lymphocytes involved in the process forced the clonality study, which revealed the presence of monoclonal rearrangement of the T-cell gamma receptor gene and the absence of amplification for B-cell receptors (Waugh et al. 2016). The diagnosis of EATL type 2 was finally confirmed.

In the present case, as in most of those already described (Brachelente et al. 2016; Nakagun et al. 2018; Noland and Kiupel 2018), the definitive diagnosis of T-cell lymphoma required the identification of clonal rearrangement for gamma T-cell receptors (TCRγ) using the PARR technique. This only differs in one studied case of nodal lymphoma in which the same pattern of immunostaining was seen despite having clonal rearrangements of a similar amplitude of both T- and B-cell receptors (cross-lineage rearrangement), when applying the molecular method for the diagnosis; in this particular case, the molecular result led to the final classification of CD3+ CD20+ anaplastic lymphoma (Nicoletti et al. 2020).

Aberrant expression of CD20 in T-cell lymphomas was also described in two cases of intestinal lymphoma associated with enteropathies in humans (Rahemtullah et al. 2008; Misra et al. 2014) and three cases in dogs (Noland and Kiupel 2018). Three hypotheses support possible causes for this co-expression in humans, but its origin and prognosis are still unclear and must be investigated in veterinary medicine. The first hypothesis supports the idea that the origin of this co-expression is a small population of T-cells that transcribe CD3 and CD20. These cells are mainly detected in peripheral blood and bone marrow and represent 3–5% of the total T-lymphocytes (Schuh et al. 2016). The second hypothesis is that CD20 expression on neoplastic T-cells is acquired during the malignant transformation of these cells. This is based on a study reporting that up to 60% of human transformed mycosis fungoides cases involve CD20 expression that was not initially present (Jullié et al. 2013). The third hypothesis then suggests a possible cross-reaction and instability of the anti-CD20 antibody, resulting in false-positive labelling of neoplastic T-cells. However, this hypothesis is considered unlikely, first because B-cells occupy their own specific territories in lymph nodes and are regularly used as positive controls, and also because this CD20 labelling of T-cells is seldom observed in the

laboratory routine, being found only in neoplastic T-cells and not in others (Sun et al. 2004). In the present case, this last hypothesis is out of the question because immunohistochemistry is performed in our laboratory on a weekly basis and the same antibody was applied to other samples at the same time without questionable results.

Type 1 enteropathy has been reported as predominant in intestinal T-cell lymphomas in dogs (Carrasco et al. 2015). The co-expression of CD3 and CD20 in this neoplasm has already been described for the species, associated with type 1 enteropathy (Noland and Kiupel 2018). To the authors' best knowledge, the present report is the first to refer to this co-expression in intestinal T-cell lymphoma associated with type 2 enteropathy, complicated by hepatic infiltration.

4. Conclusions

This case report reinforces the importance of combining different laboratory methods to accurately diagnose intestinal lymphomas and exposes a rarely reported presentation of co-expression of T- and B-cell markers in dog lymphoma, which should be considered in future investigations regarding its causes, clinical significance, and prognostic factor in the canine species.

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Conflicts of Interest: The authors declare no conflict of interest.

Chapter V

General discussion, conclusion, limitations of the study and future perspectives

1. General discussion

Hematopoietic tumors are highly prevalent in dogs, representing nearly one-third of all malignancies diagnosed annually in this species (Wilson-Robles et al. 2023). Among these, canine lymphoma is the most common and diverse, accounting for approximately 80% of hematopoietic malignancies (Valli et al. 2011; Flood-Knapik et al. 2013; Avery et al. 2014; Vail 2016). In contrast, multiple myeloma constitutes 8% of all canine hematopoietic tumors (Matus et al. 1986; Valli et al. 2017; Vail et al. 2020). Canine hematologic malignancies exhibit considerable pathogenic diversity and share genetic and treatment similarities with their human counterparts (Atherton and Mason 2022). Due to these similarities, canine lymphoma (CL) is often considered a valuable model for human non-Hodgkin lymphoma (NHL), enabling the application of human REAL/WHO classification guidelines to canine cases. This alignment facilitates comparisons between species and supports the development of effective diagnostic and therapeutic strategies for both veterinary and human oncology (Ito et al. 2014; Seelig et al. 2016; Zandvliet 2016; Martini et al. 2021). Therefore, research in this area has the potential to both contribute to veterinary oncology and be translated to human oncology.

Several aspects are deemed essential in the diagnosis and characterization of lymphoid hematopoietic neoplasms. It is well established that the diagnostic features of these neoplasms significantly impact prognosis and play a critical role in determining the most appropriate treatment for each case (Matus et al. 1986; Pavlika et al. 2008; Semedo-Lemsaddek et al. 2008; Glickman et al. 2009; Glickman et al. 2011; Semedo-Lemsaddek et al. 2016; Fernández and Chon 2018; Vail et al. 2020). In fact, the literature highlights the importance of diagnostic methods in characterizing of diferents lymphoid hematopoietic neoplams, which raises the need for advances in research into new techniques, especially with the aim of achieving a quick, early and accurate diagnosis.

Histopathological and immunohistochemical evaluations are essential for the classification and characterization of most hematopoietic lymphoid tumors in dogs, especially lymphomas. However, these methods are invasive, involve inherent risks, and often require significant time to produce results (Zandvliet 2016; Huang et al. 2024). In contrast, cytological diagnosis is faster, more sensitive, and minimally invasive, making it a popular method for diagnosing various lymphoid neoplasms. This technique is relatively safe and painless for obtaining samples (Sözmen et al. 2005; Blauvelt and Messick 2020). Despite its benefits, cytology was less effective in defining the phenotype and grade of lymphomas (Martini et al. 2022). Complementary tests may be crucial in challenging diagnostic cases, particularly for differentiating between reactive lymphocytosis and lymphoma or in cases of anaplastic round cell neoplasms, where cytology may not be conclusive (Vail et al. 2020; Wachowiak et al. 2022). As a result, there is increasing interest in additional minimally invasive techniques to complement cytology for diagnosing lymphoid hematopoietic neoplasms (Waugh et al. 2016;

Ehrhart et al. 2019; Heinrich et al. 2019; Wiley et al. 2019; Marrinhas et al. 2022; Martini et al. 2022; Valente et al. 2022; Valente et al. 2024). The objective of this study was to deepen our understanding of the use of different minimally invasive diagnostic techniques, analyzing their applicability, effectiveness and sensitivity, through the optimization and implementation of these techniques to aid the diagnosis of dogs with suspected lymphoid hematopoietic tumors.

In chapter II, the accuracy of minimally invasive diagnostic techniques (cytology, cell block, immunocytochemistry and clonality assay) was compared in dogs with clinical suspicion of multicentric lymphoma. Initially, it was observed that cytological examination resulted in the diagnosis of lymphoma in more than 80% of cases, with the majority of them being classified as high grade. This highlighted the importance of cytology as a highly suitable option to begin the diagnostic process, due to its simplicity, sensitivity, speed, affordability, safety and minimally invasive nature (Sözmen et al. 2005; Seelig et al. 2016; Blauvelt and Messick 2020). However, compared to histopathology and immunohistochemistry, prioritized by the WHO to achieve the classification of lymphomas, this approach demonstrated less consistency in determining the grade and phenotype, critical factors in the accurate diagnosis of these neoplasms (Martini et al. 2022). This indicates the need to perform additional analyzes to ensure the quality of the diagnosis in samples obtained through fine needle aspiration.

In evaluating the effectiveness of other minimally invasive techniques investigated in this study, performed on lymph node aspirate samples, the cell block and clonality assay demonstrated a relative sensitivity of 90% or more in identifying lymphomas in suspected cases, when compared to the cytological examination. These promising results were in line with existing literature (Waugh et al. 2016; Ehrhart et al. 2019; Heinrich et al. 2019) and demonstrate the importance of these techniques and their potential in diagnosing lymphoid neoplasms.

It is important to highlight that the cell block with immunocytochemistry demonstrated superiority in identifying the neoplasia and the cell type involved when compared to immunocytochemistry in cytological smears and the clonality assay. This highlights some already documented advantages of this technique, such as the ability to prepare suitable samples for subsequent immunophenotyping and detection of malignant diseases (Peleteiro 2011; Shidham 2019). The positive results achieved can be attributed, in part, to the preventive measures adopted in this study, such as the assessment of the cellularity of the cell block and the quality of the sample obtained, in addition to the use of HistogelTM to minimize possible interference in immunohistochemical studies (Shidham 2019; Melega et al. 2020). Such precautions may have contributed to obtaining excellent results with this technique.

Despite the good performance of the molecular method in identifying the neoplastic process and its phenotype, due to its complexity and higher cost, its use is recommended as a viable alternative technique after obtaining the results of the cytological diagnosis in

conjunction with immunostaining, or when other techniques are not capable of defining an accurate diagnosis, as already described (Sabattini et al. 2018; Valente et al. 2022). This study suggests the economical and time benefits of employing minimally invasive approaches in diagnosing multicentric lymphoma in dogs while maintaining the necessary diagnostic accuracy. Furthermore, it highlights the importance of a multidisciplinary approach in this diagnosis, encouraging the use of these techniques to obtain diagnostic material in a single veterinary clinical service, which also contributes to the benefits described previously.

In chapter III, the potential of the cell block technique was tested on cytological aspirates in the diagnosis of canine multiple myeloma (Valente et al. 2024). MM represents less than 1% of all malignant tumors in dogs (Valli et al. 2017). Its diagnosis is commonly based on the presence of plasmacytosis in the bone marrow, osteolytic bone lesions and high levels of myeloma proteins in serum or urine (Vail et al. 2020), but it can sometimes be challenging and complex. In the study in question, given the detection of organomegaly in the ultrasound examination, cytological analysis of the spleen was initially performed; however, it was insufficient to establish a definitive diagnosis, with hypotheses being raised of hyperplastic or neoplastic processes of lymphoplasmacytic origin. Subsequently, the evaluation of cell blocks obtained from ultrasound-guided aspiration, combined with immunophenotyping, allowed the characterization of the type of cell involved and the identification of the disseminated neoplasm in the spleen. This approach proved to be crucial in the diagnosis of dogs with MM, complementing the cytological examination and demonstrating extramedullary dissemination in two dogs, despite this being considered more common in feline MM (Patel et al. 2005).

A less invasive approach for the definitive diagnosis of canine MM generally requires a cytological evaluation of the bone marrow aspirate, with the observation of more than 20% of plasma cells to confirm this neoplasm (Vail 2016; Thompson and Dittmer 2017; Vail et al. 2020). In the absence of osteolytic lesions, this diagnosis can also be made by associating medullary plasmacytosis, the progressive increase in proteins or the identification of plasma cell clonality (Vail et al. 2020). In the present study, in the absence of evident bone lesions and in the presence of monoclonal gammopathy, immunophenotyping of cell blocks revealed approximately 70% of medullary plasma-cell density in two cases analyzed. This result confirmed the potential of this complementary technique for the definitive diagnosis of MM in dogs, especially in one of the cases, which, contrary to what was expected, showed only 16% of plasmacytoid cells in the cytological examination of the bone marrow aspirate. If it were not for the use of this innovative technique, the diagnosis of this neoplasm could have been difficult. Therefore, the use of the cell block derived from splenic and medullary cytological samples proved to be effective in assisting in the diagnosis of MM in dogs, highlighting its importance in situations with atypical presentations or even in more typical cases, helping to

confirm the diagnosis precisely. This application highlighted the significant relevance of this minimally invasive technique for further investigations.

In Chapter IV, the applicability of cytoblock and PARR was investigated in an unusual case of co-expression of T and B cell markers in a canine gastrointestinal lymphoma (Valente et al. 2022). In the present case, given the suspicion of liver disease associated with enteropathy, it was decided to perform an ultrasound-guided liver cytological examination due to the patient's weakness. Cytological evaluation of the liver was essential to investigate the possibility of GI lymphoma. Considering that this organ is frequently affected in this neoplasia, cytology can be a useful and highly sensitive tool in the diagnosis of round cell liver tumors (Coyle and Steinberg 2004; Gieger 2011; Uzal et al. 2016) and combined witht the health status of the animal, this minimally invasive approach was considered highly relevant. However, the identification of a population of small to intermediate-sized lymphocytes required complementary diagnostic tests, especially to exclude possible differential diagnosis (Uzal et al. 2016; Siegel and Wiseman 2020). With the aim of improving the accuracy of cytological diagnosis and avoiding more invasive procedures, immunocytochemistry was conducted on the cell block of the liver aspirate. This analysis revealed the co-expression of CD3 and CD20 in lymphoid cells, however, it was not possible to establish a definitive diagnosis.

Although incisional biopsies are generally recommended for histopathological evaluation in the diagnosis of enteropathies and for the differentiation of IBD from EATL type 2, due to the ability to evaluate all layers of the gastrointestinal wall (Gieger 2011; Carrasco et al. 2015), at the present report, we opted for endoscopic biopsies. This choice was mainly motivated by the fact that these biopsies are less invasive, and are especially recommended in weakened animals. Histopathological examination of these biopsies was a crucial step in confirming the diagnosis of gastrointestinal lymphoma. Based on the characteristics presented, such as the reduced size of lymphoid cells, the absence of necrosis and inflammation, the diagnosis of EALT type 2 was considered the most appropriate, as described in the literature (Uzal et al. 2016).

Immunohistochemistry of endoscopic biopsies was also performed, as it is very useful in the differential diagnosis of IBD and GI lymphoma (Carrasco et al. 2015). After the unexpected observation of the labeling of lymphoid cells with T and B cell markers, similar to what was observed in the immunocytochemistry of the liver puncture cell block, further investigation was necessary to determine the exact lineage of the lymphocytes involved in the process. For this, the clonality study was conducted through PARR, using DNA extracted from endoscopic biopsies. Clonality testing is increasingly used to distinguish inflammatory bowel disease from GI lymphoma in dogs and cats (Gress et al. 2016; Lane et al. 2018; Ohmura et al. 2018), however in this case it was used to define the cell type involved in the neoplastic process. This method revealed the presence of monoclonal rearrangement of the T cell

gamma receptor gene and the absence of amplification for B-cell receptors, defining clonality for T lymphocytes (Burnett et al. 2003; Waugh et al. 2016; Ehrhart et al. 2019). Based on these findings, it was possible to confirm the diagnosis of EATL type 2, demonstrating the co-expression of CD3 and CD20 in lymphoid neoplastic cells, a rare occurrence in canine GI lymphoma. Although the co-expression of these markers had already been described in association with EATL type 1 in dogs (Noland and Kiupel 2018), this was the first report to document such an occurrence in EATL type 2, a type of enteropathy considered less common in the species (Carrasco et al. al. 2015). The effectiveness proven by the use of these two diagnostic techniques in this case, the cell block and PARR, justified the decision to use them as low-invasive approaches in subsequent studies.

During this doctoral study, we investigated the application of innovative and less invasive techniques in the diagnosis of different types of hematopoietic tumors in dogs. When employing these techniques on samples obtained by aspiration puncture, we observed promising results, characterized by fast, accurate diagnoses and complementary to conventional diagnostic methods, thereby fulfilling the main objectives of this research project. This relevance in clinical practice is not restricted to the initial diagnosis and can extend to monitoring and early detection of recurrences. Furthermore, the information obtained played a crucial role in guiding appropriate treatments and formulating prognoses, thus highlighting the importance of these techniques in the field of veterinary oncology.

2. Conclusion

The high incidence and low cure rate of lymphoid hematopoietic tumors in dogs, especially lymphomas, characterize the need for the development of other strategies to contribute towards the management of the disease in the species and promote the survival of the animals. This doctoral project played a significant contribuition to the evolution of knowledge, relevance and application of minimally invasive techniques in veterinary medicine, directed to improve the diagnosis and contribute towards the therapy of the different types of hematopoietic tumors in dogs, such as the most prominent ones, multicentric and gastrointestinal lymphomas, and the uncommon Multiple Myeloma. Moreover, we were able to uncover an infrequently described clinical presentation of co-expression of T and B-cell markers in a T-cell lymphoma associated with type 2 enteropathy and expose an unusual expression of CD20 in canine Multiple Myeloma, which should be considered in future investigations into its causes, clinical significance and prognostic factor.

This study, in addition to highlighting the economical and temporal benefits resulting from the use of these approaches in the diagnosis and characterization of these canine neoplasms, emphasized the importance of a more efficient and accessible clinical practice for the benefit of patients and their owners. Although the need for more invasive studies is recognized for an accurate classification of this neoplasm in accordance with the REAL/WHO guidelines established in the literature, it is important to highlight that valuable information can be obtained through less invasive methods. This information, combined with clinical data, can play a crucial role in making clinical decisions for the immediate treatment of the patient and formulation of a prognosis. In short, the results achieved not only have the potential to positively impact the health of animals, but also to enrich research in this field and promote closer collaboration between veterinary oncology and human medicine.

3. Limitations of the study

This study faced some limitations, mainly related to the optimization of the techniques used and the monitoring of the animals. For example, in the PARR technique, the diversity of suggested samples and different methods for the separation and identification of DNA fragments amplified by PCR made its optimization complex. At least three different protocols were tested, but interpretation of the results was not always possible. Measures such as extraction of fresh cytological samples, assessment of the integrity and concentration of the DNA applied in the PCR reaction were adopted to ensure the clarity and quality of the results. These measurements were crucial to optimizing the molecular technique in this study.

Likewise, a pilot study was conducted to optimize the cell block technique and immunocytochemistry on cytological smears. Analysis of sample quality interference for cell blocks during this study was essential to establish standardization in the collection of representative cytological samples. For example, significant differences were observed between samples extracted with and without aspiration, due to contamination with blood. Furthermore, differences were observed between cytological samples collected from superficial lesions and from organs using ultrasound-guided punctures. A prior evaluation of the sample after histological processing was necessary to ensure its representativeness. In the immunocytochemistry technique on cytological smears, variations were made mainly in cell fixation, but it was not possible to reevaluate the cause of the inconclusive results.

Initially, the aim was to compare minimally invasive techniques to the gold standard for diagnosing these neoplasms, focusing primarily on lymphomas, and follow the animals to diagnose minimal residual disease early. However, several obstacles prevented it from being carried out, such as the impossibility of obtaining adequate samples for histopathological and immunohistochemical evaluation, as well as the failure to follow-up the diagnosed animals, which redirected the initial idea of the study towards making a comparison between techniques performed on samples obtained by fine needle aspiration in diferents lymphoid neoplasms.

4. Future perspectives

The results included here suggest that the use of minimally invasive techniques in the diagnosis of some lymphoid hematopoietic neoplasms constitute a promising strategy and should be routinely applied in clinical practice. It is essential, first of all, to improve the methodologies used. Additional research should focus on refining these techniques, aiming to further improve their diagnostic accuracy and sensitivity. Furthermore, it would also be interesting to develop new molecular diagnostic methods or identify new prognostic markers from cytological samples.

The significant accuracy and relevance observed in the results of this study highlight notable clinical applications, especially in the early diagnosis and classification of these hematopoietic neoplasms. This directly influences prognostic assessment and the selection of individualized treatments. To improve the project, it is suggested that case studies be carried out in different populations of dogs with suspected lymphoid hematopoietic tumors, expanding the applications of these techniques beyond diagnosis and providing a comprehensive view of their use in the management of theses neoplasms in the species. Advances in new diagnostic options can play a key role in improving treatment follow-up, identifying resistances, detecting minimal residual disease, and facilitating early detection of tumor recurrence, not only in dogs but also in other species of companion animals. This may also be relevant in other neoplasms observed in veterinary medicine.

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