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UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS

CÂMPUS DE JABOTICABAL

ESTUDO CLÍNICO, ULTRASSONOGRÁFICO E IMUNO-HISTOQUÍMICO DE CÃES COM LINFOMA MULTICÊNTRICO SUBMETIDOS AO TRATAMENTO QUIMIOTERÁPICO.

Michelle do Carmo Pereira Rocha

Doutoranda em Cirurgia Veterinária

Jaboticabal

2025

MICHELLE DO CARMO PEREIRA ROCHA

ESTUDO CLÍNICO, ULTRASSONOGRÁFICO E IMUNO-HISTOQUÍMICO DE CÃES COM LINFOMA MULTICÊNTRICO SUBMETIDOS AO TRATAMENTO QUIMIOTERÁPICO.

Defesa de tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – UNESP, Câmpus de Jaboticabal, como parte das exigências para obtenção do título de doutor em Cirurgia Veterinária.

Orientador: Prof. Dr. Andrigo Barboza De Nardi

Coorientadora: Profaª Drª Josiane de Morais Pazzini

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POTENTIAL IMPACT OF THIS RESEARCH

With the development of veterinary medicine and the growing social demand for pet health, there has been an increase in the longevity of dogs and cats. However, this fact has also led to a higher prevalence of chronic diseases, including cancer.

In parallel, studies involving the area of veterinary oncology, and especially translational oncology, have been gaining prominence, with increasing investments and discoveries that have been important and have implemented benefits for both humans and dogs, since dogs are an excellent experimental model and can develop a series of neoplasms spontaneously, such as lymphoma, which is similar from a molecular and genetic point of view and presents the same risk factors as Non-Hodgkin's Lymphoma.

Thus, discoveries in veterinary oncology can be applicable in the development of new therapies, such as immunotherapy and genetic and regenerative therapies, not only for dogs but also for humans.

This study provides promising results regarding the applicability of advanced ultrasound techniques in monitoring therapeutic response and determining prognosis for high-grade multicentric B-cell lymphomas. Elastography associated with clinical information, color Doppler and B-mode assessment has proven to be a good, non-invasive and inexpensive tool for monitoring therapeutic response in different phases of antineoplastic chemotherapy.

In addition, this study characterizes the immunohistochemical expression of two immune checkpoint proteins, which can serve as therapeutic targets for treating multicentric lymphoma, helping to determine which patients could respond well to blockade of these checkpoints. Immunotherapy has been developing and is providing less invasive and more effective options for treating cancer in animals. These therapies help the immune system recognize and fight cancer cells, offering an alternative to traditional treatments such as chemotherapy and radiotherapy, especially in cases of disease recurrence that do not respond well to salvage chemotherapy protocols.

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CERTIFICADO DE APROVAÇÃO

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Epígrafe

“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota”. (Madre Teresa de Calcutá)

Dedicatória

Dedico esse trabalho a Deus, a força que move o mundo e habita aqueles que nunca desistem. Dedico também aos meus pais, irmã, avós: minha família, porto seguro, inspiração e amor.

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CERTIFICADO

Certificamos que o projeto de pesquisa intitulado "**ESTUDO CLÍNICO, IMUNOISTOQUIMICO E ULTRASSONOGRÁFICO DE PACIENTES CANINOS COM LINFOMA MULTICÊNTRICO SUBMETIDOS QUIMIOTERAPIA CHOP.**", protocolo nº 1974/21, sob a responsabilidade do Prof. Dr. Andrigo Barboza Denardi, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 19 de agosto de 2021.

Vigência do Projeto	20/08/2021 a 10/02/2024
Espécie / Linhagem	Cães
Nº de animais	40 animais
Peso / Idade	Sem limites de peso e idade
Sexo	Machos e fêmeas
Origem	Rotina do Hospital Veterinário

Jaboticabal, 19 de agosto de 2021.

Fabiana Pilarski

Profa. Dra. Fabiana Pilarski
Coordenadora – CEUA

UNIVERSIDADE ESTADUAL PAULISTA
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C E R T I F I C A D O

Certificamos que o projeto de pesquisa intitulado **"Perfil imunohistoquímico de linfoma multicêntrico alto grau de imunofenótipos B e T em cães submetidos à quimioterapia CHOP provenientes do estado de São Paulo e da cidade do Porto"**, protocolo nº 8021/22, sob a responsabilidade do Prof. Dr. Andrigo Barboza de Nardi, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 16 de novembro de 2022.

Vigência do Projeto	15/03/2023 a 12/12/2023
Espécie / Linhagem	Cadelas
Nº de animais	60
Peso / Idade	Sem especificação
Sexo	Machos e Fêmeas
Origem	Rotina do Hospital Veterinário "Governador Laudo Natel" – UNESP / Jaboticabal e acervo VETPAT – Patologia Veterinária de Campinas

Jaboticabal, 16 de novembro de 2022.

Fabiana Pilarski

Profª Drª Fabiana Pilarski
Coordenadora – CEUA

LIST OF ABBREVIATIONS

ABC: ATP-binding cassette
ACL: angiocentric lymphoma
ACT: Adoptive Cell Therapy
agNOR: argyrophilic nucleolar organizing regions
AIL:angioimmunoblastic lymphoma (also known as angioimmunoblastic lymphomatous disease)
ALCL: anaplastic large cell lymphoma
Alpha-SMA: Alpha Smooth Muscle Actin
ARFI: Acoustic Radiation Force Impulse
ATL: angiotropic lymphoma
B-LCL: large B-cell lymphoma
B- or T-ALL: B- or T-acute lymphoblastic leukemia
B- or T-LBL: B- or T-lymphoblastic lymphoma
B- or T-CLL: B- or T-chronic lymphocytic leukemia
B- or T-SLL: B- or T-small lymphocytic lymphoma
CAFs: Cancer Associated Fribroblasts
CCNU: Lomustine
CG: Control Group
CGA- High Magnification Field
CEL: cutaneous epitheliotropic lymphoma
cfDNA: cell-free DNA
CHOP: (ciclophosfamide, doxorrubicin, vincristine e prednisone)
CL- Canine Lymphoma
CML- Canine Multicentric Lymphoma
CNEL: cutaneous non-epitheliotropic lymphoma
CTLA-4 – Cytotoxic T-lymphocyte-associated protein 4
DLBCL or DLBCCL: diffuse large B-cell lymphoma, cleaved or not cleaved
DLCCL: diffuse large cleaved cell lymphoma
DMAC: (Dexamethasone, Melphalan, Actinomycin and Cytarabine)
DOX: Doxorrubicin
EMC: Extracelular Matrix
F- or DSCCL: follicular or diffuse small cleaved cell lymphoma
F- or DMCL: follicular or diffuse mixed cell lymphoma; F- or DLCL: follicular or diffuse large cell lymphoma
FC: Flow Cytometry
FCCL: follicle center cell lymphoma
FDA: Food and Drug Administration
GL: Group Lymphoma
HBI: half-body irradiation
HL: Hodgkin’s lymphoma
ICIs: Immune checkpoint inhibitors
IFB: B immunophenotype group
IFT: T immunophenotype group
IHC: Immunohistochemistry
ITCL: intestinal T-cell lymphoma
Ki67: nuclear cell multiplication marker
KPT-335: (verdinexor)

Lb: lymphoblastic; Lc: lymphocytic; Cc: centrocytic; Cb: centroblastic; Ib: immunoblastic
LCIBL: large cell immunoblastic lymphoma
LGL: large granular lymphocyte lymphoma or leukemia
LLI: B-cell lymphocytic lymphoma of intermediate type
LMIs : large multivalent immunogens
LOPP: (Lomustine (CCNU), Vincristine, Procarbazine, Prednisolone)
LPL: lymphoplasmacytic lymphoma
mAbs: Monoclonal antibodies
MALT-L: mucosa-associated lymphoid tissue lymphoma
MCL: mantle cell lymphoma
MCP-1: monocyte chemotactic protein-1
MF/SS: mycosis fungoides/Sezary syndrome
MMP: matrix-metalloproteinase
MOPP: (Mechlorethamine, Vincristine, Procarbazine and Prednisone)
MRD: Minimal Residual Disease
MZL: marginal zone lymphoma
NHL: No Hodgkin's lymphoma
NLR: neutrophil-to-lymphocyte ratio
NK-CLL: NK-cell chronic lymphocytic leukemia
NMZ: nodal marginal zone lymphoma
PARR- PCR For Antigen Receptor Rearrangement
PCT: plasmacytic tumors
PCT: plasmacytoma, NOS: not otherwise specified.
PD-L1- Programmed Cell Death Ligand 1
PD-1- Programmed Cell Death 1
PET-CT: Computed Tomography (CT) with Positron Emission Tomography (PET)
PFS: Disease progression free time
P-pg: P-glycoprotein
PI: plasmacytic/plasmacytoid
PI3K-Akt: signaling pathway
PTCL: extranodal or peripheral T-cell lymphoma
RAB, Tanovea®: drug rabacfosadine
ROIs: areas of interest
SLLP: small lymphocytic lymphoma plasmocitoid
SMZ: splenic marginal zone lymphoma
SNCCL: small non-cleaved cell lymphoma
ST: Survivor Time
STAT: signaling pathway
SWV: shear wave value
TK-1: Thymidine kinase 1
VCOG: Veterinary Cooperative Oncology Group
VEGF : vascular endothelial growth factor
WBI: Whole-body irradiation
WHO- World Health Organization

ESTUDO CLÍNICO, ULTRASSONOGRÁFICO E IMUNO-HISTOQUÍMICO DE CÃES COM LINFOMA MULTICÊNTRICO SUBMETIDOS AO TRATAMENTO QUIMIOTERÁPICO.

RESUMO

O linfoma é considerado o tumor hematopoiético mais importante em cães, e se origina principalmente em órgãos linfohematopoiéticos sólidos. No entanto, também pode surgir em tecidos não linfoides, como sistema nervoso, rins e cavidade nasal. O linfoma multicêntrico é responsável por aproximadamente 80% dos casos de linfoma em caninos e pode afetar os linfonodos periféricos e cavitários, bem como o baço, o fígado, as amígdalas, a medula óssea, o sangue periférico e os órgãos extranodais, como os pulmões, os rins e o sistema nervoso central. Portanto, sua manifestação clínica varia de acordo com os órgãos afetados. A avaliação citológica é considerada uma ferramenta de diagnóstico de triagem muito útil, no entanto, o diagnóstico definitivo é obtido por meio de avaliações histopatológica e imuno-histoquímica, que permitem a definição do tipo histológico e do imunofenótipo (B ou T), respectivamente. Outros testes diagnósticos também podem fornecer o imunofenótipo, como a citometria de fluxo e PARR (PCR para rearranjo do receptor de antígeno). Vários sistemas de classificação são usados para selecionar o tratamento apropriado e avaliar o prognóstico, incluindo localização anatômica, estadiamento clínico e subestadiamento com base nas diretrizes da Organização Mundial da Saúde, características histológicas e citológicas (alto e baixo grau) e um sistema baseado em imunofenótipo (células T ou B) semelhante ao usado para classificar a doença em humanos, presença de síndromes paraneoplásicas (anemia, neutrofilia, hipercalcemia, trombocitopenia e caquexia), uso prévio de corticosteroides antes da quimioterapia de primeira linha também estão associados a um pior prognóstico da doença. Entretanto, mesmo com caracterização imunofenotípica e histopatológica semelhantes, a experiência clínica tem demonstrado que a doença pode apresentar comportamentos distintos. Por exemplo, pacientes com Linfoma Difuso de Grandes Células B (LDGCB) podem apresentar diferentes tempos de sobrevida e comportamentos com agressividade distinta. Isso torna necessário caracterizar melhor o prognóstico, descobrir novos critérios de avaliação, bem como estratégias para monitorar e determinar a eficácia das terapias antitumorais adotadas para tratamento. Devido a esse cenário, é importante a constante atualização e pesquisa em termos de monitoramento da resposta terapêutica e busca de fatores prognósticos e preditivos, a fim de permitir o desenvolvimento de terapias mais individualizadas e eficazes, aumentando a sobrevida e a qualidade de vida dos pacientes. Estudos recentes têm avaliado a aplicabilidade de ferramentas avançadas de imagem, como a elastografia ARFI, no auxílio ao diagnóstico e monitoramento da resposta terapêutica em diversas neoplasias,

sendo uma alternativa não invasiva, acessível e de fácil implementação na rotina clínica da oncologia humana e veterinária. Da mesma forma, na busca por novas possibilidades terapêuticas para tratar o linfoma de forma eficiente, diversos estudos envolvendo checkpoints imunológicos têm sido realizados em oncologia translacional, uma vez que o mecanismo de resistência à quimioterapia está relacionado à evasão do sistema imunológico em linfomas. Sugere-se que o eixo PD-1/PD-L1 e o receptor CTLA-4 podem estar relacionados à imunotolerância às células tumorais, favorecendo a carcinogênese e, portanto, podem ser um alvo terapêutico promissor para tratar a doença. O objetivo deste estudo foi avaliar a aplicabilidade do Modo-B, elastografia ARFI e expressão de PD-L1 e CTLA-4 para monitorar a resposta terapêutica e fornecer informações prognósticas e preditivas no linfoma multicêntrico canino.

Palavras-chave: Linfoma, Oncologia veterinária, Prognóstico, ultrassonografia veterinária, Imunoterapia.

CLINICAL, ULTRASONOGRAPHIC AND IMMUNOHISTOCHEMICAL STUDY OF DOGS WITH MULTICENTRIC LYMPHOMA UNDERGOING CHEMOTHERAPY TREATMENT.

ABSTRACT

Lymphoma is considered the most important hematopoietic tumor in dogs, and it originates mainly in solid lymphohematopoietic organs. However, it can also arise in non-lymphoid tissues, such as the nervous system, kidneys and nasal cavity. Multicentric lymphoma accounts to approximately 80% of lymphoma cases in canines and can affect peripheral and cavitary lymph nodes, as well as the spleen, liver, tonsils, bone marrow, peripheral blood and extranodal organs, such as the lungs, kidneys and central nervous system. Therefore, its clinical manifestation varies according to the organs affected.The cytological evaluation is considered a very useful screening diagnostic tool, however, the definitive diagnosis is obtained through histopathological and immunohistochemical evaluations, which allows the definition of the histological type and the immunophenotype (B or T), respectively. Other diagnostic tests can also provides the immunophenotype, such as flow cytometry and PARR (PCR For Antigen Receptor Rearrangement).Several classification systems are used to select appropriate treatment and assess prognosis, including anatomical location, clinical staging and substaging based on World Health Organization guidelines, histological and cytological features (high and low grade), and an immunophenotype-based system (T or B cells) similar to that used to classify human disease, presence of paraneoplastic syndromes (anemia, neutrophilia, hypercalcemia, thrombocytopenia and cachexia), prior use of corticosteroids before first-line chemotherapy are also associated with a worse prognosis of the disease. However, even with similar immunophenotypic and histopathological characterization, clinical experience has shown that the disease may present distinct behaviors. For example, patients with Diffuse Large B-Cell Lymphoma (DLBCL) may present different survival times and behaviors with different aggressiveness. This makes it necessary to better characterize the prognosis, discover new evaluation criteria, as well as strategies to monitor and determine the effectiveness of antitumor therapies adopted for treatment. Due to this scenario, it is important to constantly update and research in terms of monitoring the therapeutic response and seeking prognostic and predictive factors, in order to allow the development of more individualized and effective therapies, increasing the survival and quality of life of patients. Recent studies have evaluated the applicability of advanced imaging tools, such as ARFI elastography, in aiding diagnosis and monitoring therapeutic response in various neoplasms, being a non-invasive, accessible, and easy-to-implement alternative in the clinical routine of both human and veterinary oncology. Similarly, in the search for new therapeutic possibilities to treat lymphoma efficiently, several studies involving immunological checkpoints have been carried out in translational oncology, since chemotherapy resistance mechanism is related to

immune system evasion in lymphomas . It is suggested that the PD-1/PD-L1 axis and the CTLA-4 receptor may be related to immunotolerance to tumor cells, favoring carcinogenesis and, therefore, may be a promising therapeutic target to treat the disease. The objective of this study was to evaluate the applicability of Mode-B, ARFI elastography, and PD-L1 and CTLA-4 expression to monitor therapeutic response and provide prognostic and predictive information in canine multicentric lymphoma.

Keywords: Lymphoma, Veterinary oncology, Prognosis, Veterinary ultrasonography, Immunotherapy.

1. Introduction

Lymphomas encompass a diverse group of tumors that originate from lymphoreticular cells. They can develop in practically any body tissue, but typically affect lymphoid tissues such as lymph nodes, spleen and bone marrow [1, 2].

In dogs, lymphoma accounts for 24% of all documented neoplasms and 85% of diagnosed hematological malignancies [3]. They are classified according to their anatomical location, histologic characteristics and immunophenotype, and the most prevalent forms are: multicentric, gastrointestinal (GI), mediastinal, and cutaneous [1, 2].

Multicentric lymphoma corresponds to 84% of all Canine lymphoma (CL) [1, 2], ranging from 53-83% [4] [5], and usually displays a superficial painless lymphadenopathy [1, 2, 5].

These neoplasms occur due to clonal expansion of lymphoid cells that exhibit specific morphologic and immunophenotypic characteristics [1, 2]. Similarly to humans, B-cell CL account for 60-80% [1, 3]; T-cell correspond to 10-38%; mixed B and T-cell lymphomas represent 22% and null cell tumors (neither B nor T-cell immunoreactive) account for less than 5%.

The determination of lymphoma histologic grade is based on the number of mitoses per high power field (low grade: 0-5 mitoses/400x; medium grade: 6-10 mitoses/400x; high grade: ≥10 mitoses/400x); the architecture, growth pattern (diffuse or follicular) and immunophenotype. [1, 6, 7].

Since lymphomas stand as the most prevalent spontaneous hematopoietic tumors in dogs [8, 9], this species may be a valuable and comparable animal model for human non-Hodgkin lymphoma (NHL) research [8, 10], despite a lower occurrence of high-grade lymphomas in the latter [8].

The continuous progress in animal cancer diagnosis and treatment [9] favors the development of a more effective and individualized therapy aiming to increase patient's life quality. This review aims to describe the state of the art regarding the diagnosis and treatment of canine multicentric lymphoma (CML) from the comparative pathology point of view.

GENERAL CONCLUSION

Given the challenges of treating high-grade multicentric lymphoma in dogs, due to the extremely variable clinical behavior of this disease and the unpredictability of when and how it will relapse, this pioneering study presents ARFI elastography, combined with B-mode evaluation and clinical evaluation as a valuable tool in monitoring dogs with lymphoma before, during and after the polychemotherapy protocol. The results demonstrated significant differences in SWV values and in the ultrasound characteristics of the lymph nodes in the presence of the disease and a strong correlation with the reactivity indicated in the cytology performed serially throughout the treatment. Furthermore, the study contributed to the elucidation of new prognostic factors for the disease, since SWV values above 2.15 m/s were indicative of lymph node reactivity and associated with shorter progression-free time and shorter overall survival time. In this context, the immunohistochemical analyses performed in the search to characterize the PDL-1 and CTLA-4 marking pattern in high-grade Ts and Bs lymphomas correlating the findings with progression-free time, survival time, staging, and substaging, had results that revealed that increased production of these proteins was significantly associated with the development of immunophenotype T, and reduced survival times of affected dogs. These findings suggest that these two biomarkers may be useful and have prognostic and predictive value as therapeutic targets for multicentric lymphomas in dogs.

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