Ciência Animal Brasileira
Ciência Animal Brasileira
Brazilian Animal Science
e-ISSN 1809-6891
DOI: 10.1590/1809-6891v23e-72650E

Section: Veterinary medicine
Research article

Acute phase protein and vitamin D concentration in dogs with multicentric lymphoma

Concentração de proteínas de fase aguda e vitamina D em cães com linfoma multicêntrico

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Abstract
This study aimed to evaluate the serum concentration of vitamin D (25-Hydroxyvitamin D) and acute phase proteins (APPs; alpha-1 acid glycoprotein, haptoglobin, transferrin, ceruloplasmin, albumin, IgA, IgG and alpha-1 - antitrypsin) as potential biomarkers for prognostic and therapy response in dogs with multicentric lymphoma submitted to the CHOP (Cyclophosphamide, Doxorubicin, Vincristine and Prednisone) chemotherapy protocol. Thirteen dogs with multicentric lymphoma classified as high grade by cytology were included in the treatment group (GL), while ten healthy dogs were included in the control group (GC). Serum was collected in the weeks T0, T5 and T10 of CHOP chemotherapy protocol, for the GL group, and in a single collection, for the GC group. All the collected samples were evaluated for the APPs and vitamin D concentrations through electrophoresis and chemiluminescence methods, respectively. Diagnostic and staging tests were performed for all the dogs in the GL group, and included cytopathology, histopathology and immunohistochemistry of the affected lymph node. Of these dogs, 9 achieved a complete response and 4 a partial response to the treatment. Data analysis was performed with the R software. The results demonstrated that serum concentrations of IgA, haptoglobin and α1-acid glycoprotein were significantly different between the groups and also between the different chemotherapy times analyzed ($p<0.05$), indicating that these proteins can be considered as sensitive biomarkers for lymphoma in dogs. Furthermore, the α1-acid glycoprotein showed prognostic value for the disease, with 63% specificity. However, vitamin D concentration was not correlated with prognosis of the dogs with lymphoma.
Keywords: lymphosarcoma; prognosis; acute phase reaction; therapeutic response; hematopoietic neoplasia.

Resumo
Objetivou-se caracterizar a concentração sérica da vitamina D e das PFAs (Proteínas de Fase Aguda) (alfa-1 glicoproteína ácida, haptoglobina, transferrina, ceruloplasmina, albumina, IgA, IgG e alfa-1 – antitripsina) em cães com linfoma multicêntrico, submetidos ao tratamento quimioterápico com protocolo CHOP (Ciclofosfamida, Doxorrubicina, Vincristina e Prednisona), determinando o valor prognóstico desses marcadores para a doença. Foram avaliadas as concentrações séricas das PFAs, através do método da eletroforese e as concentrações da vitamina D, através da quimioluminescência em dois grupos experimentais, um grupo de 13 cães com linfoma multicêntrico classificados como alto grau pela citologia (GL) durante as semanas T0, T5 e T10 do tratamento com protocolo quimioterápico antineoplásico e em um grupo de 10 animais saudáveis para compor o grupo controle (GC), em coleta única. Para isso, foi realizado o diagnóstico, estadiamento e avaliação de resposta terapêutica dos 13 pacientes com linfoma multicêntrico através de técnicas de citopatologia, histopatologia, imuno-histoquímica do linfonodo periférico acometido. Foi observado que 9 pacientes tiveram resposta completa e 4 pacientes tiveram resposta parcial ao tratamento. Os dados foram analisados através do software R. Os resultados indicam que as diferenças entre as variáveis IgA, haptoglobina e α1-glicoproteína ácida foram significativas entre os grupos, e entre os diferentes momentos da quimioterapia ($p< 0,05$), indicando que podem ser marcadores sensíveis ao linfoma em cães. A α1-glicoproteína ácida apresentou valor prognóstico para o linfoma, com 63% de especificidade. Porém a vitamina D não apresentou valor prognóstico para o linfoma multicêntrico em cães.
Palavras-chave: linfossarcoma; prognóstico; reação de fase aguda; resposta terapêutica; neoplasia hematopoiética.

Received : April 26, 2022. Accepted: July 22, 2022. Published: August 24, 2022.

Introduction

Lymphomas are a diverse group of neoplasms that originate in lymphocytes [1] that are among the most common neoplasms in dogs, with an estimated annual incidence of 13-24 per 100,000 dogs in the United States[2]. In Brazil, no studies accurately indicating the incidence of the disease have been conducted to date; however, it is known that lymphoma represents 8.8% of all cancers that affect dogs, as observed during necropsy[3]. Canine lymphoma (CL) is similar to human non-Hodgkin 's lymphoma (NHL) in terms of morphological, immunophenotypic, genotypic, clinical, and prognostic characteristics, which reinforces research in medicine and pathology that compares the species, with dogs being a promising experimental model for the disease[4].

Lymphoma can be classified according to its anatomical location, with the multicentric form being the most common. This form accounts for 75% of cases. Based on cytopathological, histopathological, and immunohistochemical criteria, the most common classification is the World Health Organization (WHO) system, in which the most frequent form is diffuse large B-cell lymphoma (LDGCB). A study conducted in dogs with multicentric lymphoma in Brazil revealed that 70-80% of CL are of B-cell origin, with LDGCB being the most common subtype, representing 59.1% of cases. Additionally, the same study revealed that lymphoblastic lymphoma was the most common T-cell lymphoma, representing 11.33% of cases[5] .

The most common treatment for multicentric lymphoma is multi-drug chemotherapy; however, most treated dogs relapse and develop resistance to chemotherapy[6,7,8]. In this context, the quest to

elucidate the predisposing and prognostic factors and strategies to monitor the therapeutic response of these patients has become constant in veterinary medicine to allow intervention before the patient exhibits recurrence[5,6].

Serum vitamin D (vit D) concentrations and acute phase protein (APF) levels have been identified as prognostic and predictive factors with high sensitivity in several neoplasms, including lymphomas, in both humans and animals[9]. Also noteworthy is the presence of a receptor for 25(OH)D3 in the nucleus of neoplastic cells, the VDR receptor (recombinant vitamin D receptor protein), which suggests a potential therapeutic target [10].Conversely, APFs have already been listed as possible markers for multicentric lymphoma in dogs, including in cases of recurrence, demonstrating early changes in their concentrations. Some studies have indicated that APFs may also be considered more sensitive than the cytopathology of peripheral lymph nodes[11].

The aim of the present study was to evaluate the profile of serum concentrations of vitamin D and APFs and verify their prognostic value in dogs with multicentric lymphoma treated with the CHOP chemotherapy protocol.

Materials and methods

Thirteen canine patients that were diagnosed with multicentric lymphoma using histopathology and cytopathology were selected without consideration of breed, sex, or age. All animals underwent clinical evaluation, abdominal ultrasonography, chest radiography, blood count, and serum biochemical profile analysis, including analysis of ALT [alanine aminotransferase], FA [alkaline phosphatase], creatinine, urea, and calcium concentrations, and myelogram. These examinations were performed with the objective of evaluating the general condition of the animal, identifying comorbidities, staging the disease, and anatomical classification. The assessments were performed at the time of the initial consultation and repeated every four weeks of chemotherapy. According to the WHO, lymphoma staging involves the involvement of a lymph node (stage I), lymph nodes in a certain region (stage II), all lymph nodes (stage III), liver and spleen (stage IV), or manifestations of tumors in the blood and bone marrow (stage V) with or without clinical signs (substages B and A) [12,13].

The inclusion criteria for GL (Lymphoma Group) were cytological evaluation compatible with high-grade lymphoma and confirmation of the histological type and immunophenotype B through histopathology and immunohistochemistry. Patients with a history of recent infectious diseases (up to 1 month prior to the study), obese patients according to the nine-point body condition score (ECC) proposed previously [14], and previously treated patients - even those who received only corticosteroids - were excluded from the study.

In patients with GL, lymph nodes were collected through excisional biopsy, preferably from the popliteal lymph node, at T0 (before chemotherapy) for histopathology and immunohistochemistry tests. After collection, patients were treated with chemotherapy using the CHOP protocol, which consisted of cyclophosphamide at a dose of 250-300 mg/m^2, doxorubicin at 25-30 mg/m^2, vincristine sulfate at 0.6-0.7 mg/m- and prednisolone in decreasing doses from 2-0.5 mg/kg during the first four weeks. The complete protocol lasted 19 weeks. Whole blood collection was performed at T0 (before the start of treatment), T5 (after five weeks of treatment), and T10 (after ten weeks of treatment). These samples were centrifuged, and serum was collected and stored at -20ºC.

Ten healthy canine patients were selected for the control group (CG) and subjected to physical examination, blood count, and biochemical analyses (similar to the GL) to assess the health of these animals. While the patients in the GL group were treated, their therapeutic response was evaluated at each weekly chemotherapy session through clinical examination, measurement of peripheral lymph nodes using an electronic caliper, and through repetition of chest radiography and abdominal ultrasonography at the end of each protocol cycle that occurred every 4 weeks.

Histopathological evaluation was performed in accordance with the cytohistomorphological classification adapted from the WHO [6,15]. Immunohistochemical evaluation was performed in a partner laboratory using immunophenotyping, as well as the CD3 marker and PAX5 for T and B lymphoma, respectively. The Ki67 cell proliferation marker was also examined, and the samples were arranged on silanized slides. A healthy canine lymph node sample was used as the negative control, whereas a lymph node sample known to be positive for T lymphoma and another for B lymphoma was used as positive controls. The samples were evaluated by light microscopy, using an ocular graticule with a diameter of 26 mm(microscopy Leica DMLB Microscope, HC PLAN 10x/20, 4"x5"). The immunoexpression of CD3 and PAX5 antibodies was established by the percentage of labeled cells, while considering the number of positive cells and the total number of cells inside the graticule. This was evaluated in five fields of higher magnification, 40x [16,17].

The expression of Ki67 markers was analyzed in a 40x objective, counting the positive cells and all cells present in five random fields. These results were transformed into the percentage of positive cells, the sample being considered positive when at least 10 % of the cells exhibited immunohistochemical staining, in accordance with the methodology of Sueiro et al. (2004). The slides were evaluated using a ZEISS automatic image analyzer with the KS300-3.0 morphometric program[16,17].

To evaluate the APFs, serum protein was obtained from a polyacrylamide gel matrix containing sodium dodecyl sulfate (SDS-PAGE). Electrophoretic fractionation was performed in accordance with the modified technique described by Laemmli (1970)[18] using a vertical electrophoresis system (PROTEAN II XI-VERTICAL ELETROPHORESIS CELLS ® - BIO-RAD). In the SDS-PAGE separation of serum proteins in dogs, the proteins were analyzed using a densitometer based on the already known molecular weight of each identified serum protein.

To determine the vitamin D concentration, the laboratory assay method (Liaison 25 OH Vitamin D Total®), which uses direct competitive chemiluminescent immunoassay (CLIA) technology, was used for the quantitative determination of 25(OH)D and other hydroxylated metabolites. of vitamin D in the serum. During the first incubation, 25(OH)D dissociates from its binding protein and binds to a specific antibody in the solid phase. After 10 min, a vitamin D marker linked to an isoluminol derivative was added. After a second 10-minute incubation, the unbound material was removed with a wash cycle. Subsequently, initiating reagents were added, and a rapid chemiluminescent reaction was initiated. The light signal is measured as relative light units (RLU) using a photomultiplier, and is inversely proportional to the concentration of 25(OH)D in the calibrators, controls, or samples.

Statistical analysis was performed using R software (R Foundation for Statistical Computing, Austria). Initially, the normality of the residuals (Shapiro's test) and homoscedasticity of the variances (variance test) of all variables studied were tested. The real or transformed values of the variables studied were compared between the groups and moments using ANOVA with repeated measures in time. The differences found were identified using the Tukey test. The real or transformed values of the variables were studied in patients with lymphoma using the Student's t-test. When any variable was considered statistically significant, it was submitted to the determination of ROC (registered operative curve), which assessed the diagnostic discriminatory capacity. The same ROC test calculated the cohort point, specificity, sensitivity, and area under the test curve. Subsequently, patients classified depending on the high and low concentrations of each protein evaluated.

In the present study, a cut-off value was determined using the ROC curve method to determine the concentrations of α1-acid glycoprotein (AGP), IgA, transferrin, and haptoglobin, which were the variables that presented a p-value of <0.1 as determined using the Student's t-test; that is, they presented significant differences in the statistical analyses. Lastly, these results were compared using the Kaplan-Meier survival curve, which defines the percentage of patients who relapsed over time, that is, the disease-free time between these patients. The results are presented as the mean ± SD, and the significance was set at 5% for all tests ($p<0.05$). Multiple correspondence analysis was also performed to evaluate the results and identify the existence of an association between the variables analyzed in this study, PFA levels, and the clinical and pathological findings of the patients. For this, Statistic 7 software was used.

Results

According to the routine of (Hospital Veterinário da Universidade Estadual Paulista) Jaboticabal, SP, Brazil, 13 animals in the GL diagnosed with multicentric lymphoma through cytopathology and histopathology were included. Among the animals in the GL group, 8 (61.54%) were male and 5 (38.46%) were female; the mean for age was 6.8 years with a range of 3-14 years; and 4 (30.7%) animals were considered mixed breed, representing the majority. As for staging, no animal was classified as stage I or II, 1 (7.7%) animal was classified as stage III, 10 (76.9%) animals were classified as stage IV, and 2 (15.4%) were classified as stage V. As for the substage, two (15.4%) were classified as substage "a" and 11 (84.6%) as substage "b" (Table 1).

All the 13 cases evaluated were classified as large-cell lymphomas in the cytopathological examination. The histopathological and immunohistochemical evaluations revealed the LDGCB type was predominant, corresponding to 53.85% of the cases. T lymphoblastic lymphoma corresponded to 15.38% of the cases, immunoblastic B lymphoma to 15.38%, and lymphoma of non-specific peripheral T cells to 15.38%. An average of 16.3 mitosis figures were found in 10 higher power fields. Regarding the immunophenotype, a total of 9 patients (69.23%) had immunophenotype B, and 4 (30.76%) had immunophenotype T (Figure 1). Regarding the cell proliferation index, Ki67, an average value of 61.9%, and a mode of 70% were obtained. Patients who had a partial response to treatment had a Ki67 value of >70% (Table 1).

Table 1. Histopathological and immunohistochemical evaluation, therapeutic response, staging and sub-staging of patients in the GL group

Pacient	Histological grade	Time to relapse in days	Immunophenotype	Ki67	Staging and sub-staging
A1	Large cell diffuse lymphoma	200	B	40%	IV b
A2	Immunoblastic lymphoma	121	B	70%	IV b
A3	T lymphoblastic lymphoma	28	T	70%	IV b
A4	Peripheral T-cell lymphoma unspecified	77	T	20%	V b
A5	Large cell diffuse lymphoma	137	B	65%	V b
A6	Large cell diffuse lymphoma	65	B	45%	IV b
A7	T lymphoblastic lymphoma	353	T	90%	IV b
A8	Large cell diffuse lymphoma	461	B	85%	IV a
A9	Large cell diffuse lymphoma	Norelapse	B	15%	III a
A10	Unspecified peripheral T-cell lymphoma	No relapse	T	85%	IV b
A11	Immunoblastic lymphoma	No relapse	B	70%	IV b
A12	Large cell diffuse lymphoma	84	B	80%	IV b
A13	Large cell diffuse lymphoma	294	B	70%	IV b

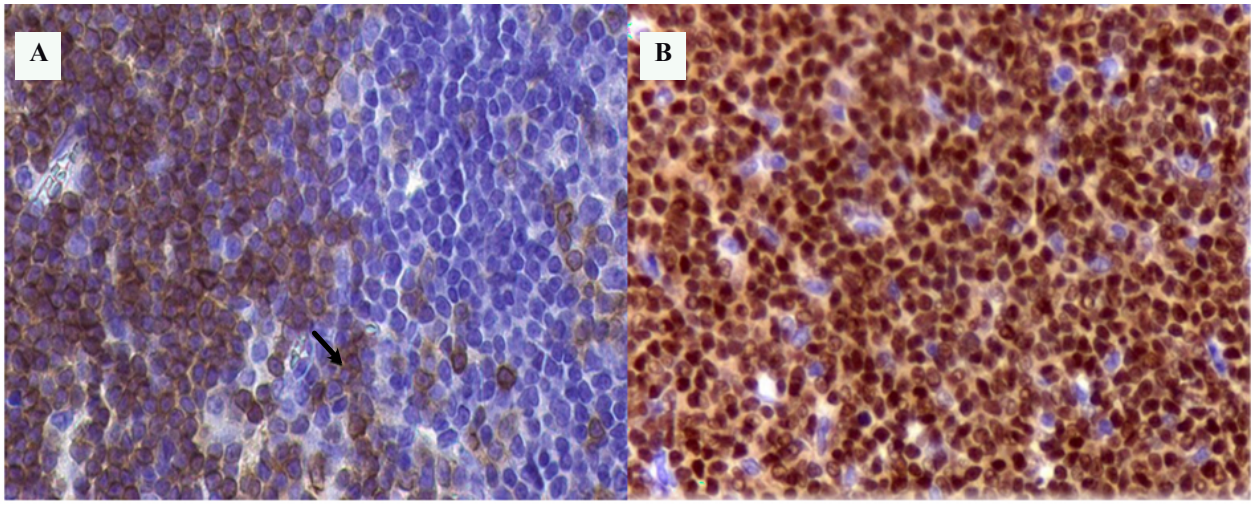

Figure 1. Photomicrograph of canine lymph node immunostaining. **A:** T-cell lymphoma, demonstrating diffuse cytoplasmic immunostaining for CD3, obj.40x., with CD3 antibody. **B:** B-cell lymphoma, demonstrating intense nuclear immunostaining for Pax5, obj.40x.

Evaluation of the response to treatment revealed that all patients showed remission of clinical signs after the first cycle of antineoplastic chemotherapy in the fifth week of the protocol, of which 9 showed a complete response and 4 showed a partial response. Evaluation of the clinical response of these patients revealed that the size of the

peripheral lymph nodes was reduced to 10 mm in diameter after the first cycle of chemotherapy. However, 46.15% of patients relapsed within 200 days after diagnosis, and 23.07% of patients relapsed more than 200 days after diagnosis (Table 1). The mean disease-free time obtained at work was 182 days (range: 28-461 days), and the mean survival time was 208.9 days (range: 73-480 days).

Regarding the assessment of vitamin D (a.k.a., 25(OH)D3), it was observed that the dogs in both the CG and GL were insufficient or deficient in vitamin D3 (Table 2); that is the average concentration of vitamin D did not differ significantly between the groups. Table 2 shows the mean values of the serum concentrations of vitamin D3, GC and GL at different times of chemotherapy treatment, demonstrating that the differences between these mean values calculated in each group and between the times of antineoplastic chemotherapy were not significant. (P >0.05).

Table 2. Means, standard deviations, and significance value of vitamin D3 concentrations (ng/mL) of dogs with multicentric lymphoma at times T0, T5 and T10 of chemotherapy and those in healthy dogs obtained using the CLIA chemiluminescence technique

Variable	Group	Time	Mean	SD	P-value	
D3	Control	T0	45,93	26,39	0,1133	**a**
	Lymphoma	T0	32,77	13,74	0,1133	**a**
		T5	29,79	14,69		**a**
		T10	**30,49**	**12,77**		**a**

Equal letters indicate that values do not differ from each other. SD: standard deviation. Unit of measure: ng/ml. Reference values for the species: deficiency, up to 24.9 ng/mL; insufficiency, 25-99.9 ng/mL; sufficiency, 100–120 ng/mL; elevated, >150 ng/mL.

Analysis of serum concentrations of APFs, revealed 18-31 protein fractions in healthy dogs and those with lymphoma, but only 11 fractions showed evidence in electrophoretic tracing. The total serum proteins included albumin, alpha globulins, beta globulins, and gamma globulins. The alpha globulin subfractions found in this assay included alpha-1-antitrypsin (AAT), AGP, alpha-2ceruloplasmin, and alpha-2-haptoglobin. Transferrin was found to be a betaglobulin. The IgA and IgG levels were also measured. Statistical analyses performed using ANOVA showed significant differences related to the presence of the disease and the evolution of antineoplastic therapy between the means of IgA and α1-acid glycoprotein, with values differing from each other (p< 0.05).

GL patients had higher levels of IgA (Figure 2 A), AGP (Figure 2B), and haptoglobin (Figure 2 C) in the first week of treatment, that is, before starting chemotherapy, at T0, compared with that of patients in GC. Furthermore, only the mean values of AGP and IgA concentrations decreased throughout chemotherapy in weeks T5 and T10 (Table 4). The haptoglobin concentration showed a significant difference with the presence of the disease (p<0.05), that is, when comparing the CG and GL group at week T0; however, this did not vary significantly during the chemotherapy treatment (Tables 3 and 4).

Table 3. Means, standard deviations, and significance values of PFA concentrations (mg/dL) in of dogs with multicentric lymphoma and healthy dogs, obtained using SDS-PAGE

Variable	Group	Moment	Mean	SD	P Value	Letter
IgA	**Control**	**T0**	**16,33**	**4,971**	**0,0118**	**a**
	Lymphoma	**T0**	**37,58**	**21,38**		**b**
CERULOPLASMIN	**Control**	**T0**	**26,3**	**15,91**	**0,166**	**a**
	Lymphoma	**T0**	**35,95**	**17,66**		**a**
TRANSFERRIN	**Control**	**T0**	**249,7**	**63,3**	**0,3575**	**a**
	Lymphoma	**T0**	**307,4**	**109,5**		**a**
ALBUMIN	**Control**	**T0**	**3926**	**260,8**	**0,4418**	**a**
	Lymphoma	**T0**	**4372**	**1356**		**a**
A1 ANTITRYPSIN	**Control**	**T0**	**185,6**	**37,11**	**0,0645**	**a**
	Lymphoma	**T0**	**270,9**	**128**		**a**
IgG	**Control**	**T0**	**1320**	**193,9**	**0,1909**	**a**
	Lymphoma	**T0**	**1542**	**737,4**		**a**
HAPTOGLOBIN	**Control**	**T0**	**108,7**	**56,15**	**0,0003**	**a**
	Lymphoma	**T0**	**418,9**	**275,4**		**b**
AGP	**Control**	**T0**	**11,14**	**3,066**	**0,0247**	**a**
	Lymphoma	**T0**	**52,03**	**35,67**		**b**

Equal letters indicate that the values do not differ from one another. SD: standard deviation, p-value: significance value.

Table 4. Means, standard deviations and significance value of PFA concentrations in dogs with multicentric lymphoma in (mg/dL) at times T0, T5, T10 of antineoplastic therapy obtained using SDS-PAGE

Variable	Group	Moment	Mean	SD	P Value	Letter
IgA	**Lymphoma**	**T0**	**37,58**	**21,38**	**0,0118**	**b**
		T5	**29,67**	**14,72**		**ab**
		T10	**26,58**	**10,24**		**ab**
CERULOPLASMIN	**Lymphoma**	**T0**	**35,95**	**17,66**	**0,166**	**a**
		T5	**38,64**	**21,1**		**a**
		T10	**45,49**	**23,54**		**a**
TRANSFERRIN	**Lymphoma**	**T0**	**307,4**	**109,5**	**0,3575**	**a**
		T5	**333,6**	**150,2**		**a**
		T10	**330**	**133,4**		**a**
ALBUMIN	**Lymphoma**	**T0**	**4372**	**1356**	**0,4418**	**a**
		T5	**4136**	**889,4**		**a**
		T10	**4644**	**1380**		**a**
A1 ANTITRYPSIN	**Lymphoma**	**T0**	**270,9**	**128**	**0,0645**	**a**
		T5	**303,3**	**109,8**		**a**
		T10	**260,2**	**98,76**		**a**
TOTAL IgG	**Lymphoma**	**T0**	**1542**	**737,4**	**0,1909**	**a**
		T5	**1121**	**531,8**		**a**
		T10	**1154**	**489,8**		**a**
HAPTOGLOBIN	**Lymphoma**	**T0**	**418,9**	**275,4**	**0,0003**	**b**
		T5	**608,5**	**353,3**		**b**
		T10	**644,6**	**349,7**		**b**
AGP	**Lymphoma**	**T0**	**52,03**	**35,67**	**0,0247**	**b**
		T5	**43,03**	**38,39**		**ab**
		T10	**43,97**	**32,73**		**ab**

Equal letters, values do not differ from each other. The results are presented as the mean ± D, and the significance was set for all tests at 5% (p<0.05). SD: Standard deviation; P-value: significance value.

Graphs 2A, 2B, and 2C demonstrate that dogs with lymphoma had a greater dispersion of individual IgA, AGP, and haptoglobin concentration values in relation to the mean compared with that of dogs in the GC, indicating a strong correlation between the disease and alterations in the concentration of the variable in question. In the same way, graph 2A demonstrates that the dispersion of IgA values in the GL was greater than in the CG, with the values differing statistically from each other.

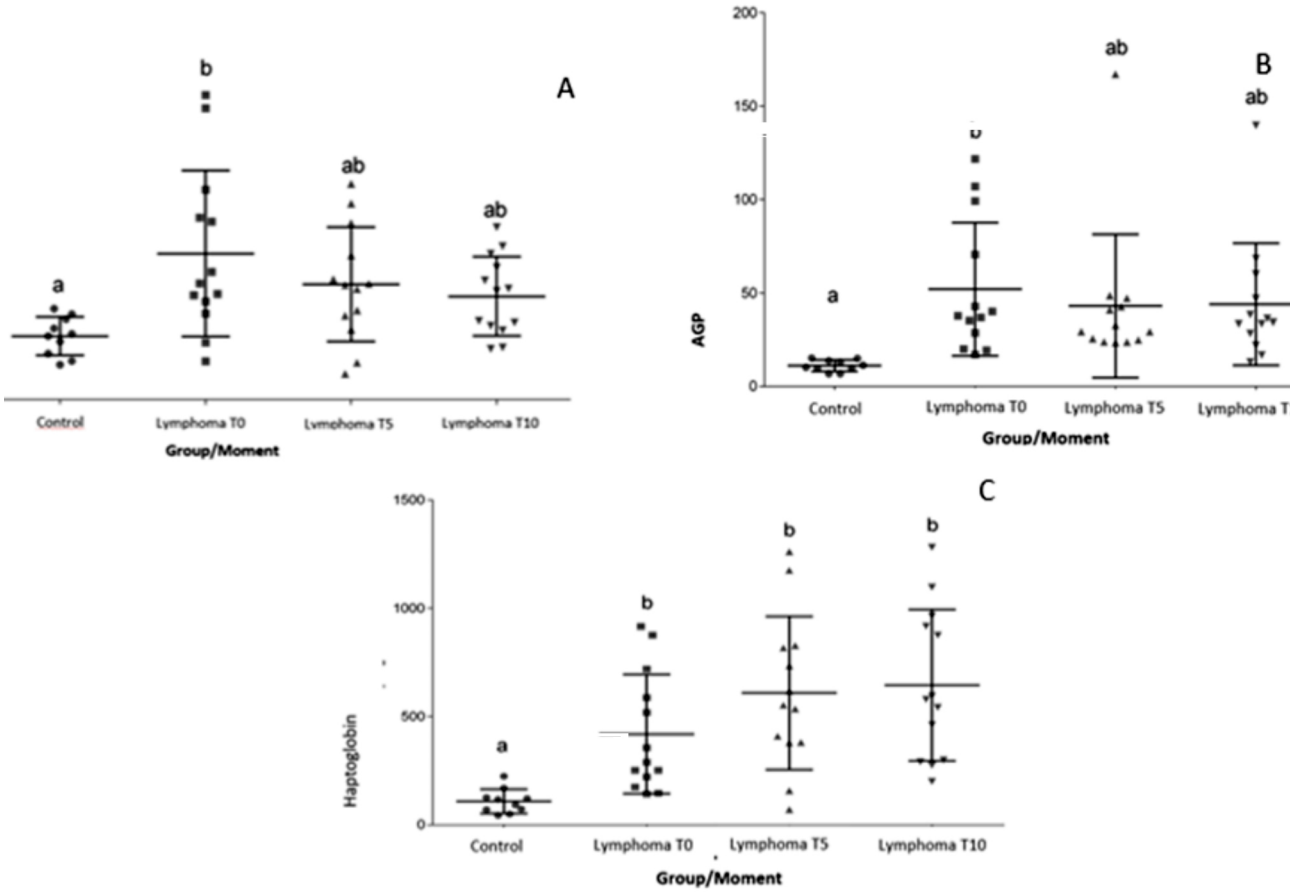

Figure 2. (A): Dispersion between IGA concentration values in mg/dL of dogs with Lymphoma during chemotherapy treatment and in the control group. Comparison of IGA concentrations between the GL at weeks T0, T5 and T10 of chemotherapy treatment and the GC. There was a significant difference by the t test (p = 0.0118). **(B):** Dispersion between the values of haptoglobin concentration in mg/dL of patients with Lymphoma during chemotherapy treatment and in the control group. Comparison between haptoglobin concentrations between the LG at weeks T0, T5 and T10 of chemotherapy treatment and the GC. There was a significant difference by the t test (p=0.0003). **(C):** Comparison between the concentrations of α1-acid glycoprotein during the chemotherapy treatment and between the experimental groups (CG: Control Group and LG: Lymphoma Group). Comparison of AGP concentrations between the GL at weeks T0, T5 and T10 of chemotherapy treatment and the GC. There was a significant difference by the t test (p=0.0247).

Patients with higher α1-acid glycoprotein concentrations at the time of diagnosis had longer times to relapse and longer survival. A cut-off value for the concentration of α1-acid glycoprotein at the time of diagnosis was determined, from which a distinction was observed from the perspective of survival and disease-free time of these patients. Patients with a serum α1-acid glycoprotein concentration of >34.8 mg/dL (Table 5) at the time of diagnosis took longer to relapse than patients with a serum concentration lower than this value at T0, according to can be observed in the graph in Figure 3, obtained from analysis using the Kaplan-Meier curve method (Figure 3).

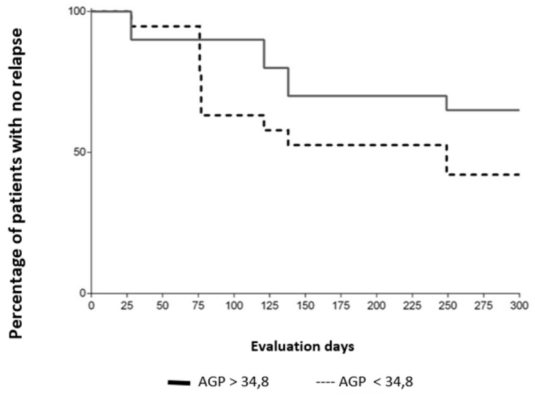

Figure 3. Recurrence curves over time of patients with lymphoma with a serum concentration of α1-acid glycoprotein greater than and less than 34.8 mg/dL. Survival curves of dogs with Lymphoma with "low" alpha-1-acid glycoprotein and "high" alpha-1-acid glycoprotein. There was a significant difference by the analysis of ROC curves and the Kaplan Meier test (p=0.0304).

The sensitivity and specificity of the concentration of α-1-acid glycoprotein in dogs with multicentric lymphoma were 61% and 62%, respectively. The probability of recurrence in patients in a cutoff was 35%, whereas the value of recurrence in patients without a cutoff was 58%, with a P-relapse of 0.139 and a P-value of 0.0232 (Table 5).

Table 5. Comparison between patients with multicentric lymphoma who relapsed before 200 days (6/13), with a mean disease-free time of 182 days, and those who did not relapse (7/13) within the study time interval

Variable	Relapse	Mean	SD	P Value	P- Prognosis	Coorte value
IGA	N	30,84	15,84	0,0866	0,9999	
	Y	31,78	17,41			
CERULOPLASMIN	N	43,99	17,69	0,2014	0,1213	
	Y	35,4	23,5			
TRANSFERRIN	N	291,3	119,3	0,0906	0,09103	
	Y	361,5	133			
ALBUMIN	N	4629	1180	0,1764	0,2258	
	Y	4098	1225			
A1 ANTITRIPSIN	N	276,1	101,1	0,9034	0,8658	
	Y	280,5	125,3			
TOTAL IGG	N	1217	711,8	0,5476	0,2599	
	Y	1337	482,2			
HAPTOGLOBIN	N	592,7	315,9	0,0484	0,3981	
	Y	516,1	360,1			
AGP	N	55,94	42,03	0,0232	0,0304	<34,8
	Y	35,15	20,18			
VITAMIN D3	N	29,87	15,07	0,5722	0,5447	

Equal letters: values do not differ from each other. The results are presented as mean ± SD, and the significance was set at 5% (p<0.05) for all tests. SD: Standard deviation; P-value: significance value.

Discussion

The findings regarding the classification of lymphomas in the model adapted from the WHO corroborate what is described in the literature, where the LDGCB type appears to be the most common histological type in the USA, Europe, and Brazil [19,20]. This histologic subtype is the most common type among non-Hodgkins lymphomas in humans, as demonstrated by human studies in oncology as well [20].

Immunophenotype B is predominant in the literature, representing approximately 70% of cases of multicentric lymphoma. Additionally, high-grade T lymphoma had a worse prognosis and shorter survival time in several studies [12,15]. This is because the T immunophenotype is associated with numerous paraneoplastic syndromes such as hypercalcemia, a lower treatment response, and an increased recurrence rate [12,15]. However, discordantly, Frantz et al. (2012)[21] pointed out a much closer relationship between the immunophenotypes, with 55% and 45% of the cases attributed to the B and T phenotypes, respectively. Regarding Ki67, this marker was positive in all means, with a high percentage value of 61.9%, which demonstrates a high proliferative cell index, corroborating the findings that classify lymphomas as high grade.

The treatment responses of the patients in the present study were evaluated at the fifth and tenth weeks of treatment in accordance with studies by Vieira (2013)[22]. The tumor response to treatment was determined in the fifth week of the CHOP protocol due to the greater likelihood of dogs with multicentric lymphoma experiencing complete remission after the first instance of doxorubicin administration in the protocol; however, Hernandez et al. (2017)[23] stipulated the tenth week for the observations, as this period better indicates the activity of doxorubicin in patients. During the clinical response, a reduction in the size of the lymph nodes to their normal size (<10 mm) was observed, according to a study by Dobson et al. (2001)[24] , who determined that this characterizes complete response, as well as the disappearance of clinical signs in patients in this period and the absence of alterations in imaging tests, abdominal ultrasound and chest X-rays. In studies by Vieira (2010)[25], serum PFA concentration is generally averaged as the concentration of patients' serum proteins in patients with higher serology in any group compared to the control group, corroborating other authors who argue that dogs with inflammatory and neoplastic diseases exhibit elevated globulin and PFA production [26,27].

Additionally, according to Vieira (2010) [25] a significant increase in serum alpha-1-acid glycoprotein (AGP) concentrations (p<0.05) was also reported in the presence of lymphoma, but it also associated with a

significant increase in transferrin and IgG concentrations. However, the findings described in the present study (Tables 2 and 3), did not find significant differences between the variables IGG, transferrin, albumin, ceruloplasmin and alpha-1-antitrypsin between the experimental groups, and between studies such as weeks did not occur during treatment ($p > 0.05$). They can influence the action of doses, suggesting that at any time of collection, patients are not sufficiently stimulated to influence the action of doses, as explained by Cerón et al. (2005)[28]. Immunophenotype B and collection at the beginning of disease progression may influence the concentration of APFs in the serum of these patients who tend to have an acute phase reaction a little later, suggesting the collection and measurement of APFs at more intervals so that there is an even more reliable assessment[28]. According to Gahmberg et al., (1978)[29] AGP can be produced by lymphocytes, justifying its high protein content in a controlled form with lymphoma [28].

No statistically significant differences related to the concentrations of the analyzed serum proteins were observed between the chemotherapy times. However, a reduction in the concentrations of IgA, IgG, and alpha-1-antitrypsin was observed in the tenth week of treatment (Table 3). This finding was justified by the action of the antineoplastic agent doxorubicin, which can cause lesions in the hepatic and renal parenchyma, leading to hypoproteinemia and proteinuria[30].

Haptoglobin (Hb) is one of the most important APFs in all species. In dogs with cancer, Calazans et al. (2009)[26] compared the PFA profile in the serum of healthy dogs and dogs with multicentric lymphoma. They detected an increase in ceruloplasmin and haptoglobin, as well as a reduction in albumin, in dogs with lymphoma[26,28,31]. In addition, another study by Battisti et al. (2013)[32] evaluated APFs in dogs with mammary neoplasia and detected elevated haptoglobin and decreased albumin concentrations in bitches with ulcerated tumors compared with the control group, suggesting that this may be a prognostic factor for this type of neoplasia[32]. According to Cerón et al. (2005)[28], haptoglobin can already be identified in the circulation in the first 24 h after injury, and its concentration increases considerably, reaching a peak in four days. Thus, it may be a sensitive marker for early lymphoma and other neoplasms in dogs[28].

IgA is found in high concentrations in cases of infectious disease, connective tissue disease, liver disease, myeloma, and other tumors of the reticuloendothelial system. IgA is present at a lower concentration in fetuses, newborn animals before colostrum ingestion, and animals with immunological deficiencies. Hematopoietic disorders often induce globulinemia, which justifies its increase in the LC in the pre-treatment period, and may indicate recurrence in these patients[33]. These findings suggest that AGP is a protective factor for multicentric lymphoma in dogs, where dogs with higher serum AGP content at the time of diagnosis are less likely to experience recurrence or have longer blood-free interval. disease compared to dogs with lower AGP concentrations.

These results concur with those of Garnica et al. (2020)[8], in which AGP was proven to be a natural anti-inflammatory agent that inhibits neutrophil activation and increases the secretion of the interleukin-1 antagonist receptor through macrophages, thus inhibiting the proliferation of lymphocytes in cases of lymphoma, primarily in neoplastic patients[8]. Calazans (2009)[26] and Eckersall (2008)[34] reported that determining AGP concentrations can prove useful when estimating tumor burden by comparing dogs with lymphoma treated with or without doxorubicin. Furthermore, elevation in the serum concentration of this protein has been described in dogs with lymphoma, carcinomas other than mammary carcinoma, and sarcomas[28]. AGP was significantly elevated ($p<0.05$) in dogs with lymphoma at T0, corroborating findings in the literature and suggesting that determining AGP concentrations may be useful in estimating the tumor burden in dogs with lymphoma treated with doxorubicin and those without treatment[26,34].

However, AGP is one of the main APFs, especially in felines, that can increase its serum concentration by approximately 1000 times from baseline levels in malignant neoplastic or acute/chronic inflammatory processes. This parameter should be included as a differential marker for the evaluation of neoplasms or acute inflammatory processes when assessing dogs and cats[28]. The results of the Kaplan-Meier curve analysis differed from those observed by Dias et al. (2017)[23], in which no correlation was observed between higher AGP concentrations and an improved multicentric lymphoma diagnosis in dogs. For these authors, only lower concentrations of transferrin and albumin were associated with a lower disease free time (TLD) and shorter patient survival time[23].

Similar to the present study, the study by Vieira et al. (2010)[25] also noted a substantial increase in AGP concentration in canine patients with multicentric lymphoma compared to the CG, suggesting that these patients had an exacerbated acute phase reaction at the time of serological collection. However, even though the prognostic value of this protein was not evaluated in the aforementioned study, it was observed that patients with higher levels of this protein had greater survival. These results differ from those reported by Dias et al. (2015)[23], in which no correlation was observed between higher concentrations of alpha-1-acid glycoprotein and an improved multicentric lymphoma prognosis in dogs. In this study, it was found that only the lowest

concentrations of transferrin and albumin were associated with lower TLD and shorter patient survival time[23].

Although few studies have been conducted using dogs, the association between low serum concentrations of Vitamin D and the risk of developing neoplasms has been described in dogs with different types of cancer, including lymphomas[35,36]. Patients with serum levels below 24.99 ng/mL are considered to have deficient vitamin D levels, and patients with serum levels of 25-99 ng/mL are considered vitamin D insufficient. ng/mL and above 99 ng/mL are considered sufficient[37]. In the GC, 80% of the animals were insufficient, 10% sufficient and 10% were deficient, with a concentration range of 25.3-115.2 ng/mL and a mean value of 45.93 ng/mL. The 25(OH)D concentration range obtained in the GL population at T0 was 11.2-62.1 ng/mL with a mean value of 32.77 ng/mL. Regarding the different weeks of chemotherapy, 23.07% of patients had vitamin D3 deficiency at T0, 46.15% patients were deficient at T5, and 38.46% patients were deficient at T10.

The results regarding vitamin D were similar than those for study by Wakshlag et al. (2011)[38] who correlated low vitamin D3 levels with an increased incidence of mast cell tumors in Labrador retrievers; these animals had a mean serum vitamin D3 concentration of 36.7 ng/ml. In addition, a previous study demonstrated an important correlation between vitamin D3 levels and the incidence of numerous neoplasms such as lymphomas, hemangiosarcomas, carcinomas, and sarcomas (Husbanda B, 2013)[39]. Vitamin D3 is believed to regulate cell proliferation, maturation, and apoptosis, thus influencing the development of autoimmune, cardiovascular, and mainly neoplastic diseases[14].

The findings of the present study, in which the mean values calculated in each group did not differ significantly, nor did it between the moments of antineoplastic chemotherapy (P >0.05), suggest that lower serum vitamin D levels are not a risk factor for the development of multicentric lymphoma in dogs; although the mean concentration differed between the groups, this was not significant in the statistical analysis. Similar results were observed in studies by Sánchez-Céspedes et al. (2018)[40], in which the serum vitamin D3 concentration was compared between bitches with mammary tumors and dogs without carriers (males and females), and no differences were found that would indicate a relationship between vitamin D3 concentration and mammary carcinogenesis. In contrast, Selting et al. (2014)[37] reported a strong correlation between serum vitamin D3 levels and the development of cancer in dogs, with animals with optimal vitamin D3 levels (100-120 ng/ml) showing a drastic reduction in the incidence of cancer in cases of soft tissue sarcoma.

Conclusions

The serum proteins IgA, alpha-1-acid glycoprotein, and haptoglobin may be considered as sensitive markers to provide diagnostic support in cases of multicentric lymphoma in dogs; however, further studies are needed to evaluate the prognostic value of these markers in a larger number of animals. Therefore, APFs can work as indicators of lymphoma in dogs, but not in isolation, as long as clinicians have an indicative clinical picture. Alpha-1-acid glycoprotein has shown prognostic value in multicentric high-grade lymphoma in dogs and is a protective factor in patients with the disease. Furthermore, the vitamin D3 level measured in patients undergoing chemotherapy was not an effective prognostic marker in patients with multicentric lymphoma classified by cytology as high-grade and immunophenotype B, which corresponded to the predominant type of lymphoma in this study.

Conflict of interests

The authors declare no conflicts of interest

Author contributions

Conceptualization: M.C.P. Rock*; Research*: M.C.P. Rocha, J. M. Pazzini and A.B. DeNardi. *Methodology*: M.C.P. Rock; T.K. Garnica, H. Fukumasu and F.A.R. Sueiro. *Project management:* M.C.P. Rocha, J. M. Pazzini and A.B. DeNardi. *Data curation*: R. A. R. Uscategui. *Writing (original draft):* M.C.P. Rock. *Writing (review & editing):* J. M. Pazzini and F. N. de Paiva. *Supervision*: A.B. DeNardi.

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