Hematological and biochemical profiles of canine CD45− T lymphomas are different from other immunophenotypes

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Abstract

Background: Canine multicentric lymphomas are lymphoproliferative malignancies that have increased in recent decades. The patient's treatment and prognosis are determined by the grade, histological type, and lymphoma immunophenotyping.

Aim: To investigate the paraclinical signs and survival time in canines with different lymphoma immunophenotypes.

Methods: Over 2 and a half years, 47 untreated dogs were diagnosed with multicentric lymphoma at the Veterinary School Hospital of Uruguay. The disease was clinically and cytologically diagnosed, and immunophenotyping was determined by flow cytometry. After the immunophenotyping, most of the patients were grouped into the following: B (LB), T aggressive (LTCD45+), or T-zone lymphoma (LTCD45−). The patients' haematological values, calcemia, lactate dehydrogenase (LDH) levels, and plasmatic electrophoretic profiles were all determined immediately after that.

Results: Of all canine lymphomas, 55.3% were B, 31.9% were LTCD45+, and 10.6% were TCD45−. Only 2.2% were classified as nonB/nonT, and survival time differed between groups. Patients with LTCD45− lymphomas had a mean life span of 641 days after diagnosis, followed by LB (166 days) and LTCD45+ (62 days). Red blood cell count, hematocrit, and hemoglobin levels did not differ between groups. However, the LTCD45− group had significantly higher lymphocyte levels than the LTCD45+ and LB groups ($p = 0.01$ and 0.006, respectively). Levels of albumin, alpha-1, and alpha-2 globulins did not differ between groups. On the other hand, gamma globulins levels in the LTCD45− were higher than in the other lymphoma groups. The presence of hypercalcemia and high plasma LDH levels were associated with patient severity. Only the TCD45+ group had hypercalcemia although both the LB and TCD45+ groups had elevations in LDH activity. Interestingly, there was a direct relationship between high LDH values (greater than 500 IU/l) and lower survival in TCD45+ lymphomas.

Conclusion: Survival time and hematological and biochemical patterns differed among canine lymphomas immunophenotypes. Patients of LTCD45− phenotype showed higher lymphocyte counts and gamma globulin levels and more prolonged survival. Serum LDH activity may provide additional prognostic information in high-grade T-cell lymphoma.

Keywords: Dogs, Immunophenotype, Multicentric lymphoma, Paraneoplastic syndromes, Survival times.

Introduction

The incidence of malignant lymphomas, both in humans and canines, has increased in the last decades. In dogs, they represent between 7% and 24% of all neoplasms (Vail *et al.*, 2013) and 80% of hematopoietic tumors (Ponce *et al.*, 2010; Aniolek *et al.*, 2014). It most often affects middle-aged or elderly dogs (Dorn *et al.*, 1967; Merlo *et al.*, 2008), and the most predisposed breeds are Doberman, Rottweiler, Boxer, and Bernese Mountain Dog (Comazzi *et al.*, 2018). Lymphoma has multifactorial causes, but genetic or environmental factors increase its incidence (Breen and Modiano, 2008; Marconato *et al.*, 2009; Pastor *et al.*, 2009).

Patient survival depends on the type of lymphoma and the degree of disease progression. Most canine lymphomas arise from B-cells and are usually characterized by the expression of CD21 and CD79a (Ruslander *et al.*, 1997; Valli *et al.*, 2011; Ernst *et al.*, 2016; Pawlak *et al.*, 2016). A smaller proportion are T-cell lymphomas, identified by the expression of CD3 and CD5 markers and, depending on the T-cell subtypes, by CD4 or CD8 markers (Avery *et al.*, 2014). In addition, there is a small portion of lymphomas that do not express labeling for T or B cells, identified as null cell lymphoma (Ponce *et al.*, 2010). In general, high-grade T-cell lymphoma has a worse prognosis

than high-grade B-cell lymphomas (Kiupel *et al.*, 1999; Beaver *et al.*, 2010; Papakonstantinou *et al.*, 2013; Aresu *et al.*, 2015). However, low-grade T-cell lymphoma, overrepresented by T-zone lymphoma (TZL), shows a better prognosis and a less aggressive clinical course. This variation of T-cell lymphomas represents about 10%–15% of the T-cell phenotype (Ponce *et al.*, 2004; Valli *et al.*, 2006; Avery *et al.*, 2014; Jark *et al.*, 2020). Canine small clear cell / TZLs are associated with the absence of the CD45 marker (Seelig *et al.*, 2014). These are defined histopathologically by small or intermediate cells with low mitotic index. Lymphocytes have a "hand mirror form" that enlarges the paracortex and medullary cords while leaving the nodal architecture intact (Ponce *et al.*, 2004; Valli *et al.*, 2011). CD45⁻ T-cell lymphoma generally showed aberrant immunophenotypes, with CD4⁻CD8⁻ being the most frequent (Pawlak *et al.*, 2016; Harris *et al.*, 2020). There is little information on why CD45⁻ patients have better survival. The CD45 marker is considered a hallmark in diagnosing the disease (Martini *et al.*, 2015, 2017).

Hematological and biochemical alterations have been observed in patients with lymphoma. Anemia and thrombocytopenia have been associated with poor prognosis (Abbo and Lucroy, 2007; Miller *et al.*, 2009). Meanwhile, it was reported that canines with neutrophilia aggravate the evolution of the disease (Gavazza *et al.*, 2008; Perry *et al.*, 2011; Calvalido *et al.*, 2016; Curran and Thamm, 2016). Mature lymphocytosis is a common finding in TZL (up to 50% of dogs) but does not affect prognosis (Seelig *et al.*, 2014; Martini *et al.*, 2016). As suggested, this could be due to the overflow of nodal lymphocytes into the bloodstream, as the loss of CD45 renders galectin unable to bind, evading apoptosis activation and prolonging lymphocyte survival (Hernandez and Baum, 2002; Seelig *et al.*, 2014; Martini *et al.*, 2016). Hypercalcemia and gammopathies are paraneoplastic syndromes frequently associated in dogs with lymphomas (Vail *et al.*, 2013). Also, increased lactate dehydrogenase (LDH) activity suggests a poor prognosis and has been associated with the presence of relapses (Zanatta *et al.*, 2003; Marconato *et al.*, 2010). Although the paraclinical parameter provides us with non-specific information, it allows the clinician to assess the severity of the disease. The prognosis of canine lymphoma depends on many factors, but the immunophenotype of the lymphoma is one of the most important (Marconato *et al.*, 2011; Valli *et al.*, 2013). The median survival of canines with indolent T-cell (low-grade) lymphoma is up to three times that of high grade B- or T-cell lymphomas (Valli *et al.*, 2013). Unfortunately, unlike in humans (non-Hodgkin lymphoma), there are no veterinary prognostic scoring systems routinely used for canine lymphoma (Fontaine *et al.*, 2017). Despite this, some biochemical parameters have been studied with controversial results in search of additional prognostic value.

This study aimed to determine whether the hematologic or biochemical changes observed in dogs with lymphoma are related to their immunophenotypes (B, TCD45⁺, and TCD45⁻) or to their survival time.

Materials and Methods

Clinical cases of canine lymphoma

A total of 47 untreated dogs seen at the Veterinary School Hospital of Uruguay between April 2018 and September 2020 with a clinical diagnosis of multicentric lymphoma were included in this study. The lymphoma diagnosis was confirmed by cytologic evaluation of the enlarged lymph nodes, while flow cytometry performed lymphoma immunophenotyping. All dogs were at least clinical stage III, except for one dog with LTCD45⁺, stage II. Unfortunately, chest X-rays, abdominal ultrasound, and bone marrow aspirations were not available in all cases, which did not accurately determine stages IV and V lymphoma. The time from cytologic diagnosis to death or euthanasia was defined as their overall survival time (OST).

Data collected from patients included breed, age, sex, reproductive status, genetic history, clinical examination findings, laboratory test results, cytologic examination, and imaging.

Cytology and immunophenotyping

Cell samples

Lymph node aspirates performed in each patient were used to confirm the cytologic diagnosis and to study the immunophenotype. Fine needle aspiration obtained punctures from the popliteal lymph nodes using a 5 ml disposable syringe and a 21-23G hypodermic needle. A clinical pathologist performed cytologic evaluation by May-Grunwald Giemsa stained smears. For immunophenotyping, lymph node aspirates were placed in phosphate-buffered saline and EDTA (2 nM, pH 7.4) at room temperature until use (no more than 2 hours) (Jalla *et al.*, 2004).

Sample staining and data collection

Cell samples were counted, and the final volume was adjusted to $0.5–1×10^7$ cells/ml. Propidium iodide (10 μg/ml–Invitrogen from Thermo Fisher Scientific) was used to determine cell viability, and only samples with vitality greater than 80% were included in the research. The lymphocytes were then incubated with 5 μl of each of the monoclonal antibodies conjugated to different fluorochromes in a dark place at room temperature for 45 minutes according to the manufacturer's recommendations (BIORAD, CA). One ml of red cell lysing solution (QUICKLYSIS, Cytognos, Salamanca, Spain) was added to the samples and incubated for 10 minutes at room temperature. The panel of antibodies used to identify the cell line is shown in Table 1. All samples were analyzed in duplicate on a Becton Dickinson (BD) Accuri C6 flow cytometer (BD Biosciences, CA). An unstained tube was used as a negative control (Martini *et al.*, 2020; Ferrari *et al.*, 2021).

For data collection, a lymphocyte gate was defined based on the complexity of the cytoplasm and cell dimension, depicted on the forward-scatter plot

Table 1. Monoclonal antibodies used for differentiation and labeling of canine lymphocytes by flow cytometry obtained from patients with multicentric lymphoma.

Antibody	Fluorochrome	Clone	Manufacturer	Specificity
CD3 Mouse anti dog	FITC	CA17.2A12	BIORAD	Thymocytes and mature T lymphocytes
CD4 Rat anti dog	FITC	YKIX302.9	BIORAD	Neutrophils and T helper lymphocytes
CD5 Rat anti dog	FITC	YKIX322.3	BIORAD	Thymocytes and T lymphocytes
CD8 Rat anti dog	RPE	YCATE55.9	BIORAD	T cytotoxic lymphocytes
CD21 Mouse anti dog	RPE	CA2.1D6	BIORAD	Mature B lymphocytes
CD34 Mouse anti dog	RPE	1H6	BIORAD	Haematopoietic stem cells
CD45 Rat anti dog	APC	YKIX716.13	BIORAD	Pan leucocyte

FITC: Fluorescein isothiocyanate; RPE: R-phycoerythrin; APC: Allophycocyanin.

versus side scatter to allow good visualization of the different cell populations contained in the sample. A debris threshold was established to analyze the data, and a minimum of 5,000 cells was counted within the lymphocyte gate of each sample, as suggested by Lana *et al.* (2006). Parameter analysis was performed using a bandpass filter of 533/30 nm for FITC-conjugated antibodies (FL1), 585/40 nm for PE-conjugated antibodies (FL2), and 675/25 nm for the APC fluorophore (FL4). Single staining controls were used to calculate color compensation. The FL1 detector was corrected by subtracting a percentage of 4.75% from FL2, and the compensation value for the FL2 channel was 9.10%, deducted from FL1. The data were analyzed with CFlow Plus software (BD).

Hematological and biochemical determinations
Blood from dogs was collected by cephalic vein puncture. Samples were placed in tubes anticoagulated with EDTA (K3 plus ethylenediamine tetraacetic acid) and in dry tubes. Cellular blood variables [red blood cell (RBC), hemoglobin, hematocrit, white blood cell (WBC), neutrophils, lymphocytes, monocytes, eosinophils, platelet counts] were performed with a veterinary hematology analyzer (Mythic 18 Vet, Orphée, Geneva, Switzerland), and by microscopic evaluation of air-dried smears stained with May-Grunwald Giemsa. Biochemical analyses (calcemia, LDH activity, total proteins, and albumins) were performed on serum samples in a semiautomatic spectrophotometer (CB 350i-Wiener lab Group, Rosario, Argentina). To ensure that hypercalcemia was not due to lipemia or hemolysis, it was interpreted in relation to the serum albumin level, using a correction formula as suggested by Bergman (2012): adjusted calcium (mg/dl) = [Calcium (mg/dl)–albumin (g/dl)] + 3.5. The reference ranges used for these variables were from Oregon State University. Gammopathies were explored by capillary electrophoresis (Minicap, Sebia, Barcelona, Spain). The protein fractions were albumin and alpha 1, alpha 2, beta and gamma globulins.

Ethical approval
The Ethical Committee approved this investigation of the Faculty of Veterinary Medicine of *Universidad de la República* (University of the Republic) of Uruguay (CEUA FVET-801 111900-000780-19). In addition, the owners of the dogs gave their signed consent for the participation of their animals in this study.

Statistical analysis
Analyses were performed with the statistical program PAST 4.03. Normal distribution was tested by the Shapiro–Wilk test. When the variables presented a normal distribution, the group effect (LB *vs.* LTCD45$^+$ *vs.* LTCD45$^−$) on hematological and biochemical variables was analyzed by one-way analysis of variance, and post hoc comparisons (differences between groups) were performed with Tukey's test. When the variables did not have a normal distribution, a Kruskal–Wallis test was performed to evaluate the group effect. The Mann–Whitney test was used to compare groups.

The proportions of the different hematological and biochemical abnormalities were analyzed according to the immunophenotype using Fisher's exact tests. Kaplan–Meier curves were drawn and compared by the Long–Rank (Mantel–Cox) test to assess the influence of immunophenotype and LDH activity above and below 500 IU/l in LTCD45$^+$ and LB phenotypes on overall survival. The prognostic value of LDH was not investigated in the LTCD45$^−$ subgroup because none of the dogs had LDH levels above 500 IU/l. Each step represents the death of individual cases. OST was calculated in days from the time of diagnosis by cytology to the time of death or censor (460 days) and expressed as hazard ratio (HR). The statistical software used was GraphPad Prism (GraphPad Software, 2365 Northside Dr., Suite 560, San Diego, CA 92108). Differences were considered significant when $p \leq 0.05$.

Results

Patient population and immunophenotyping
Of the 47 dogs with multicentric lymphoma, 22 were males (46.81%), and 25 were females (53.19%). The

Table 2. Population characteristics and immunophenotyping by flow cytometry of neoplastic populations identified in lymph node aspirates from 47 dogs with multicentric lymphoma.

ID	Sex	Reproductive status	Breed	Age (years)	CD45	CD34	CD21	CD3	CD5	CD4	CD8	Phenotype
1	F	I	Bichon Frise	7.5	+	−	+	−	−	−	−	B
2	F	I	French Bulldog	5	+	−	+	−	−	−	−	B
3	F	N	Crossbreed	9	+	−	+	−	−	−	−	B
4	F	N	Crossbreed	12	+	−	+	−	−	−	−	B
5	F	N	Crossbreed	8	+	−	+	−	−	−	−	B
6	F	N	Poodle	8	+	−	+	−	−	−	−	B
7	F	N	Cocker	8	+	−	+	−	−	−	−	B
8	F	N	Labrador	8	+	−	+	−	−	−	−	B
9	F	N	Rottweiler	4	+	−	+	−	−	−	−	B
10	F	N	Bloodhound	8	+	−	+	−	−	−	−	B
11	F	N	German Shepherd	7	+	−	+	−	−	−	−	B
12	F	N	Golden Retriever	4	+	−	+	−	−	−	−	B
13	F	N	Golden Retriever	9	+	−	+	−	−	−	−	B
14	M	I	Cocker	10	+	−	+	−	−	−	−	B
15	M	I	Bullmastiff	6.5	+	−	+	−	−	−	−	B
16	M	I	Dachshund	6	+	−	+	−	−	−	−	B
17	M	I	English Bulldog	6	+	−	+	−	−	−	−	B
18	M	I	Golden Retriever	10	+	−	+	−	−	−	−	B
19	M	I	Golden Retriever	9.5	+	−	+	−	−	−	−	B
20	M	I	Golden Retriever	10	+	−	+	−	−	−	−	B
21	M	I	Golden Retriever	5	+	−	+	−	−	−	−	B
22	M	N	Crossbreed	11.5	+	−	+	−	−	−	−	B
23	M	N	Crossbreed	10	+	−	+	+	−	−	−	B
24	M	N	Pitbull	6	+	−	+	−	−	−	−	B
25	M	N	Doberman	8	+	−	+	−	−	−	−	B
26	M	N	Golden Retriever	6	+	−	+	−	−	−	−	B
27	F	I	Boxer	9	+	−	+/−	+	−	+		T CD45+/CD4+
28	F	I	Golden Retriever	4.5	+	−	−	+	+	+	−	T CD45+/CD4+

29	F	N	Crossbreed	6	+	−	−	+	+	+	−	T CD45+/CD4+
30	F	N	Boxer	8	+	−	−	+	−	+	−	T CD45+/CD4+
31	F	N	Boxer	8	+	−	−	+	+	+	−	T CD45+/CD4+
32	F	N	Boxer	2.5	+	−	−	+	+	+	−	T CD45+/CD4+
33	F	N	Labrador	11	+	−	−	+	+	−	+	T CD45+/CD8+
34	F	N	Dogue de Bordeaux	8	+	−	−	+	−	+	−	T CD45+/CD4+
35	M	I	Poodle	8	+	−	−	+	+	−	+	T CD45+/CD8+
36	M	I	Labrador	10	+	−	−	+	−	−	+	T CD45+/CD8+
37	M	N	Boxer	7	+	−	−	+	+	+	−	T CD45+/CD4+
38	M	N	Boxer	7.5	+	−	−	+	+	−	+	T CD45+/CD8+
39	M	I	Labrador	8.5	+	−	−	+	+	−	−	T CD45+ CD4− CD8−
40	M	I	Airdale Terrier	8	+	−	−	+	+/−	+/−	−	T CD45+ CD4− CD8−
41	M	N	Labrador	9	+	+/−	−	+	+	+/−	−	T CD45+
42	F	N	Beagle	7	−	−	−	+	+	−	−	T CD45− CD4− CD8−
43	F	N	Yorkshire	14	−	−	−	+	+	−	−	T CD45− CD4− CD8−
44	F	N	Bullmastiff	6	−	−	−	+	+	−	−	T CD45− CD4− CD8−
45	M	I	Golden Retriever	8	−	−	−	+	+	−	−	T CD45− CD4− CD8−
46	M	I	Golden Retriever	10	−	−	−	+	+	+	−	T CD45− CD4+
47	M	I	Golden Retriever	8	+	−	−	−	−	−	−	No B/ No T

F: female; M: male; N: neutered; I: intact; +: indicates positivity in more than 65% of the neoplastic cells; −: indicates negativity in more than 65% of the neoplastic cells; +/−: indicates positivity in more than 20% and less than 65% of the neoplastic cells.

mean age of dogs with lymphoma was 7.87 years (range 2.5 to 14 years), and the most represented breeds were Golden Retriever (23.4%), Boxer, Labrador, and crossbreeds (12.8% each, respectively), (Table 2).

While 55.3% of the dogs were large B-cell lymphomas, 42.6% belonged to the T-cell immunophenotype. Within the T-cell lymphomas, 75% were CD45+ (15/20) and 25% were CD45− (indolent TZLs). Most of the CD45+ T-cell lymphomas were CD4+ (53.3%), the 26.7% were CD8+, and 13.3% CD4/CD8 double negative. One dog (6.7%) classified as CD45+ T (CD3+, CD5+) had weak CD4 expression (marking positivity in more than 20% and less than 65% of neoplastic cells) and was not included in any of the above subgroups.

The CD45− T-cell group represented 10.64% of the total patients and consisted of small cells in the flow cytometry scatter plots. 80% (4/5) of the dogs were CD4/CD8 double negative, and the other 20% were CD4+. Interestingly, the mean age of this group at diagnosis was 9 years, higher than that of the B and T phenotypes (7.7 and 7.6 years, respectively).

In this study, only one dog was classified as non-B and non-T-cell lymphoma, as it did not show positive marking to any of the specified lineages (Table 2).

The chemotherapy treatment that each patient received after diagnosis depended on their treating clinician and the economic possibilities of their owners, with a wide variety of treatments for all phenotypes. Single-drug regimens (glucocorticoids, doxorubicin, lomustine, vincristine, and chlorambucil) as well as combos and multidrug protocols were used (Cyclophosphamide, vincristine, and prednisone or Cyclophosphamide, doxorubicin, vincristine, and prednisone). A total of 13

Table 3. Summary of blood cell counts and biochemical parameters in lymphomas of different phenotypes.

	B	T CD45⁺	T CD45⁻	Reference interval
Number of cases	26	15	5	
Erythrocytes (x10⁶/µl)	6.2 ± *1.1*	5.8 ± *1.6*	5.8 ± *1.7*	5.5–8.5
Hematocrit (%)	41.8 ± *7.7*	39.4 ± *13*	38.2 ± *9.3*	37–55
Hemoglobin (g/dl)	15 ± *2.5*	14 ± *4.2*	14.1 ± *3.8*	12–18
Leucocytes/µl	17,985.4 ± *15*,723.6	13,054.5 ± *8*,267.4	24,020 ± *11*,923.6	6,000–17,000
Lymphocytes/µl	3,912.4 ± *6*,242.2	2,632.9 ± *1*,790	13,270 ± *11*,995.7	1,000–4,800
Neutrophils/µl	13,056 ± *10*,546.6	10,059 ± *7*,694	9,733 ± *864.8*	3,000–11,400
Monocytes/µl	491.9 ± *311.8*	332.3 ± *274.5*	715.4 ± *230.1*	150–1,350
Eosinophils/µl	517.6 ± *707.3*	136.1 ± *141.5*	300.6 ± *599.6*	100–750
Platelets/µl	275,130 ± *139*,873	224,000 ± *78*,100	354,500 ± *190*,176	200,000–900,000
Calcium (mg/dl)	9.5 ± *1*	9.9 ± *3.2*	9.7 ± *0.47*	8–11.4
Lactate dehydrogenase (IU/l)	525.2 ± *327.3*	414.4 ± *321.8*	212.6 ± *81.7*	30–236
Total proteins (g/dl)	6.4 ± *0.74*	6.4 ± *1.15*	7.5 ± *0.76*	5.4–7.6
Albumin (g/dl)	3.4 ± *0.58*	3.1 ± *0.74*	3.3 ± *0.22*	3.1–4.2
Globulin (g/dl)	2.98 ± 0.65	3.2 ± 0.92	4.2 ± 0.89	2.3–3.4
Alpha 1 (g/dl)	0.31 ± 0.08	0.35 ± *0.14*	0.28 ± *0.05*	0.1–0.4
Alpha 2 (g/dl)	0.86 ± 0.39	0.91 ± 0.29	0.8 ± 0.08	0.4–1.0
Beta (g/dl)	1 ± 0.43	1.3 ± 0.60	1.45 ± 0.41	0.5–2.4
Gamma (g/dl)	0.59 ± 0.18	0.68 ± 0.45	1.55 ± 0.82	0.3–1.2

Results presented as means ± standard deviation (SD).

dogs did not receive treatment (LB, *n* = 7; LTCD45⁺, *n* = 3; LTCD45⁻, *n* = 3).

Hematologic variables
RBC counts, hematocrit, and hemoglobin concentration did not differ between canines with different lymphoma phenotypes (Table 3). In this study, 13 of 47 dogs with lymphoma had anemia. The distribution and degree of severity of anemia did not differ between groups.

WBC counts were significantly different between T lymphoma subtypes. Dogs with LTCD45⁻ lymphomas had higher leukocyte counts than with LTCD45⁺ (p = 0.036). However, no significant differences in WBC counts were found between the other groups. Of the patients with lymphoma, 27.7% had leukocytosis, but the distribution between the groups was similar. Only two of the patients belonging to the LTCD45⁺ group had leukopenia.

Lymphocyte counts did not differ between the LTCD45⁺ and LB groups. However, the LTCD45⁻ group had significantly higher lymphocyte levels than the LTCD45⁺ and LB groups (p = 0.01 and =0.006, respectively) (Fig. 1A). Canines with lymphocytosis were identified in 20% of cases. Sixty percent of the patients belonging to the LTCD45⁻ group had mature lymphocytosis. On the other hand, 20% of the dogs had lymphopenia, and its distribution was similar between the groups.

Mean eosinophil counts were higher in dogs with LB than those in the LTCD45⁺ group (p = 0.049), but there was no difference with the LTCD45⁻ group. There were also no differences between the LTCD45⁺ and LTCD45⁻ groups. Eosinophilia was identified in 15.2% of patients. Eosinopenia was seen in 36.96% of patients with lymphoma, and the highest proportion was observed in the LTCD45⁻ group (p = 0.027).

Mean monocyte counts were significantly different between groups (p = 0.026). The LTCD45⁻ group had more circulating monocytes than the LTCD45⁺ group (p = 0.03) but did not differ from the LB group. There were also no differences between LB and LTCD45⁺ groups (Fig. 1B). Monocytopenia was shown in 21.74% of lymphoma patients and had a similar distribution between groups.

There were no significant differences in neutrophil and platelet counts between either group. Neutrophilia and thrombocytopenia were observed in 26.1% and 32.5% of patients, respectively. No differences were found between the groups.

Serum biochemistry
Calcium levels were similar between groups. Hypercalcemia was observed in 4 of the 38 patients
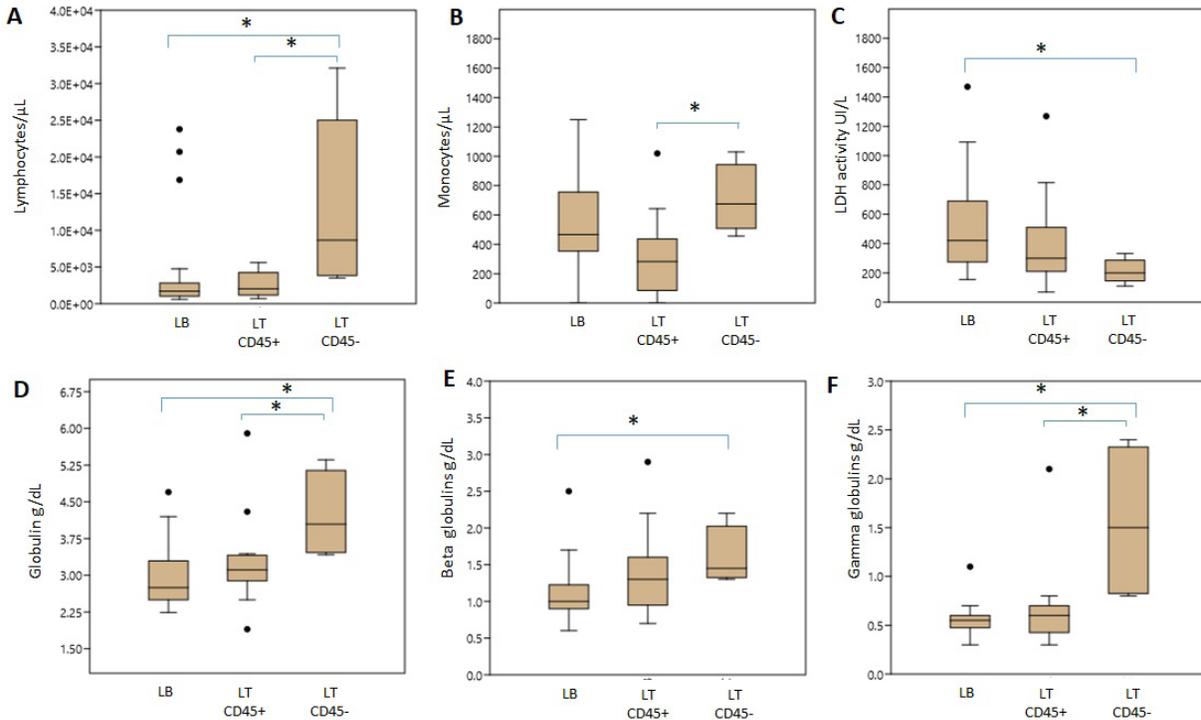
Fig. 1. Differences in blood cell counts and biochemical variables between LB, LT CD45$^+$ and LT CD45$^-$ groups. A. Lymphocyte count. B. Monocyte count. C. LDH activity. D. Globulins. E. Beta globulins. F. Gamma globulins. Data are plotted as box-and-whisker plots and show outliers. Data are presented as means +/− SD. Asterisk indicates differences on the same graph, $p < 0.05$.
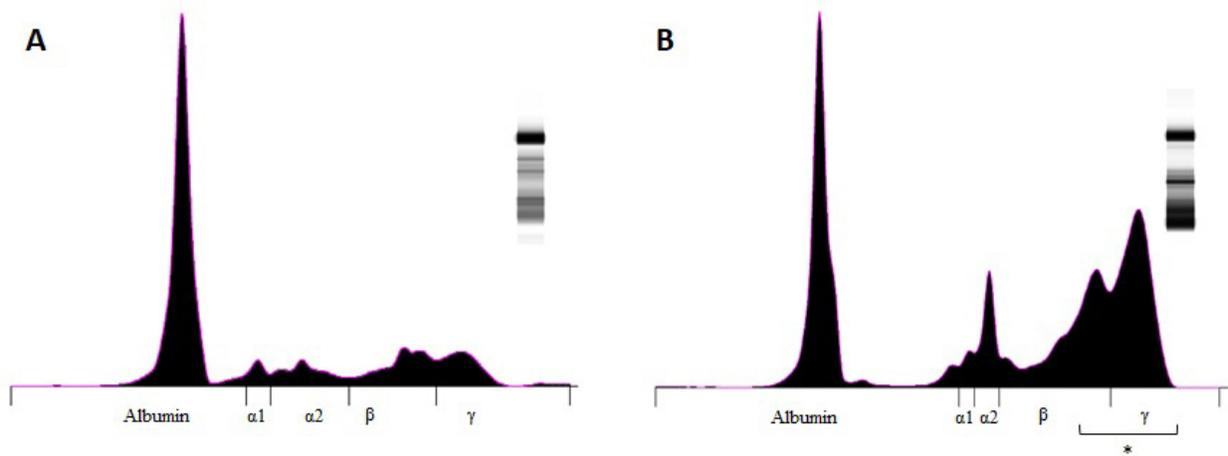
Fig. 2. Graphic representation of the capillary protein electrophoresis (A, LB; B, LTCD45$^-$). The different protein fractions are observed (albumins; alpha-1 globulins, alpha-2 globulins; beta globulins, gamma globulins). A polyclonal gammopathy is distinguished, characterized by the presence of a broad-based beta-gamma peak (*).

with lymphoma, and all of them belonged to the LTCD45$^+$ group ($p = 0.018$). Mean LDH activity differed significantly between groups ($p = 0.029$), being significantly higher in LB than in LTCD45$^-$ ($p = 0.012$), while there was no difference between the other groups

(Fig. 1C). Increased LDH activity was found in 74.4% of patients. Moreover, 83.3% of LB, 69.2% of LTCD45$^+$ and 40% of LTCD45$^-$ had increased LDH activity.
Plasma total protein concentrations were similar between groups. Also, when the protein fractions were
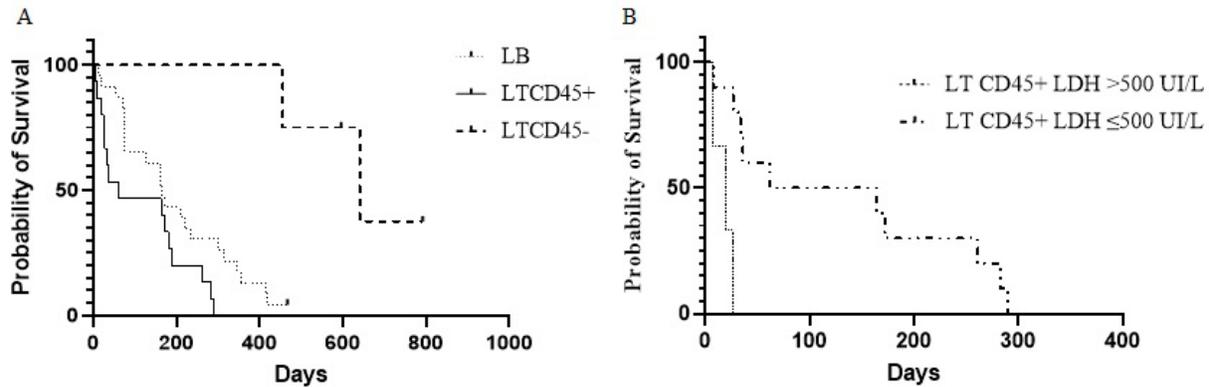
Fig. 3. Kaplan–Meier curves depicting overall lymphoma survival in 42 dogs divided into phenotypic groups (LB, *n* = 23; LTCD45⁺, *n* = 15; LTCD45⁻, *n* = 4), (A); and in 13 dogs with LTCD45⁺ and different LDH activity (>500 IU/l, *n* = 3; ≤500 IU/l, *n* = 10), (B). "I", live patients.

studied, the mean levels of albumin, alpha-1, and alpha-2 globulins did not differ between groups (Table 3). However, LTCD45⁻ had higher levels of gamma globulins than the LB and LTCD45⁺ groups (*p* = 0.004 and 0.01, respectively), (Figs. 1D, F, and 2) and higher levels of beta globulins than the LB group (*p* = 0.02), (Fig. 1E).

Survival

OST differed between groups (*p* = 0.0004). LTCD45⁻ dogs survived longer than LTCD45⁺ and LB (*p* = 0.001 and 0.0025, respectively), and in turn, LB dogs had a longer survival time than LTCD45⁺ (*p* = 0.034) (Fig. 3A). The median survival time was 641 days (95% CI: 455–792) for LTCD45⁻, 166 days (95% CI: 14–418) for LB and 62 days (95% CI: 7–290) for LTCD45⁺. At the end of the study, one of the dogs in LB and two of the dogs in LTCD45⁻ survived.

The HR was 0.51 (95% CI: 0.25–1.07) between LB and LTCD45⁺, 5.72 (95% CI: 2.6–13.29) between LB and LTCD45⁻, and 6.45 (95% CI: 2.48–16.73) between LTCD45⁺ and LTCD45⁻.

Decreased survival time was detected for LTCD45⁺ dogs with LDH activity above 500 IU/l compared to those with values below 500 IU/l (*p* = 0.0018) (Fig. 3B). The median survival time was 113 days (95% CI: 69–437) for LDH values below 500 IU/l and 20 days (95% CI: 7–26) for values above 500 IU/l, and the HR was 5.44 (95% CI: 0.47–62.6). No significant differences were detected in survival time for LB dogs with LDH activity above or below 500 IU/l (*p* = 0.30).

Discussion

Proper diagnosis of lymphomas allows veterinary clinicians to evaluate the best treatment and management of a disease that severely affects patient survival and well-being. In the present work, we explored the association between different immunophenotypes of lymphomas and paraclinical signs. This information could help us to understand or predict the development of the disease.

Of the 47 dogs with multicentric lymphoma in the study, B-cell phenotype was diagnosed in 55.3% of the cases. In contrast, 42.5% of the lymphomas were of T-cell origin (10.64% TZL and 31.9% aggressive T lymphoma). Most of the previous works showed a high prevalence of phenotype B over T lymphomas (Teske *et al.*, 1994; Ponce *et al.*, 2010; Valli *et al.*, 2011; Valli *et al.*, 2013). However, the reported percentage of T-cell lymphomas in canines differs between regions. For example, European and North American studies showed the incidence of T-cell lymphomas to be approximately 30% (Fournel-Fleury *et al.*, 1997; Ruslander *et al.*, 1997; Calvalido *et al.*, 2016; Pawlak *et al.*, 2016). On the other hand, studies from several groups in Latin America found T immunophenotypes equal to or higher than those reported in the present study (Moreno and Bracarense, 2007; Álvarez-Berger *et al.*, 2009; Neuwald *et al.*, 2014). However, a recently published Brazilian paper reported frequencies similar to European and North American studies (Jark *et al.*, 2020). The genetic background of dog populations and environmental factors could contribute to these regional differences (Modiano *et al.*, 2005; Pastor *et al.*, 2009; Ruple *et al.*, 2017). Golden Retrievers and Boxers were the most common breeds in our research, which matches what has previously been reported in America (Modiano *et al.*, 2005; Jark *et al.*, 2020). Not surprisingly, Golden Retrievers were overrepresented in the LTCD45⁻ group due to a notable predilection of the breed to develop TZL (Seelig *et al.*, 2014; Hughes *et al.*, 2018).

In the present work, 25% of the T lymphomas were classified as small clear cell-TZL due to the cytological appearance and the presence of TCD45⁻ lymphocytes

by flow cytometry (Seelig *et al.*, 2014; Martini *et al.*, 2016). Although aggressive T lymphomas were prevalent (75%), the importance of the diagnosis of TCD45⁻ lymphoma is based on its better prognosis and less aggressive clinical course (indolent subtype) (Ponce *et al.*, 2004; Valli *et al.*, 2006; Valli *et al.*, 2013; Avery *et al.*, 2014). Little is known about why CD45⁻ T lymphomas are the least aggressive of the lymphomas and the evolution of clinical and paraclinical parameters in patients. CD45 is a T lymphocyte accessory molecule that stabilizes the interaction between T and B cells and plays a vital role in the maturation of the thymocytes (Ledbetter *et al.*, 1993). CD45-null transgenic mice showed a reduced ability to mature CD4⁻ CD8⁻ T cells into CD4⁺ CD8⁺ T cells (Byth *et al.*, 1996), which could explain why most patients in the CD45⁻ T cell group were also CD4⁻ CD8⁻ on flow cytometry. Several authors previously reported this aberration (Avery *et al.*, 2014; Seelig *et al.*, 2014; Martini *et al.*, 2015; Pawlak *et al.*, 2016).

One-third of all patients with lymphoma had anemia or leukocytosis. Several authors listed anemia as a side effect of chronic disease (Marconato *et al.*, 2013; Valli *et al.*, 2013; Parachini-Winter *et al.*, 2019). Any of the lymphoma groups showed differences in severity. However, dogs with LTCD45⁻ lymphomas had higher leukocyte counts than LTCD45⁺. Interestingly, patients with CD45⁻ T-cell lymphomas had higher levels of lymphocytes than patients with B-cell and CD45⁺ T-cell lymphomas, and 60% of TCD45⁻ patients had mature lymphocytosis. Previous studies have shown that mature lymphocytosis is a common finding in indolent TZLs, exceeding 50% of dogs in all cases (Flood-Knapik *et al.*, 2013; Avery *et al.*, 2014; Seelig *et al.*, 2014; Martini *et al.*, 2016). The lack of significant difference in leukocyte count with the LB group was possibly due to the low number of animals in the LTCD45⁻ group.

Due to the lack of TCD45, knockout mice showed an increase in splenic B cells, mainly of the B1 population (Byth *et al.*, 1996). In addition, it has been reported that CD45⁻ cells could evade, at least in part, the process of apoptosis, reaching a longer lifespan (Hernandez and Baum, 2002; Seelig *et al.*, 2014; Martini *et al.*, 2016). The highest proportion of eosinopenia was found in CD45⁻ T lymphomas. To the authors' knowledge, there are no reports of eosinopenia in lymphomas, although there are reports of eosinophilia in intestinal T lymphomas related to paraneoplastic IL-5 synthesis (Marchetti *et al.*, 2005; Ozaki *et al.*, 2006). However, monocytic cell counts were higher than those of CD45⁺ T lymphomas. It was previously reported that dogs with lymphoma had higher monocyte counts than healthy animals (Perry *et al.*, 2011; Calvalido *et al.*, 2016). However, to the authors' knowledge, this would be the first study to analyze monocyte counts among T-cell lymphoma subtypes.

Despite not being statistically significant, total protein levels were striking higher in patients with LTCD45⁻. Possible reasons for the lack of significant effect of total protein in our study are a small number of animals analyzed in the LTCD45⁻ group. Albumin and alpha-1 and alpha-2 globulin levels did not differ between the groups. However, gamma globulin levels in LTCD45⁻ were higher than in the other lymphoma groups. Although this finding may be paradoxical, it has been described in several B- and T-lymphocyte disorders in humans (Mims, 2018). This profile could be related to the differences found in circulating lymphocyte levels and the role of CD45 in regulating the immune system. However, few reports describe variations in protein fractions, and none of them consider immunophenotype (Gavazza *et al.*, 2009; Tappin *et al.*, 2011).

A patient's survival depends on the type of lymphoma, the degree of disease progression, and the treatment. Although this work has the limitation of not having standardized treatments among lymphoma immunophenotypes, OST differs between groups. LTCD45⁻ dogs survived longer than the other groups (mean 641 days), while LB dogs (mean 166 days) had a longer survival time than LTCD45⁺ (62 days) (Fig. 3). The lack of CD45 co-stimulatory T molecules and the potential secondary stimulation of innate responses could explain, at least in part, the low aggressiveness and their classification as indolent T lymphomas. Of note, the OST of our dogs with lymphoma was lower than that reported in the literature (Ruslander *et al.*, 1997; Ponce *et al.*, 2004; Valli *et al.*, 2013; Curran and Thamm, 2016). This could be explained by the fact that many dogs with aggressive lymphoma were not treated or were treated with an inadequate protocol.

High-grade T lymphomas are the most aggressive. T cells (CD4⁺ and CD8⁺) play an important role in the coordination of the immune system, and their deficiency could severely affect the patient's health. Hypercalcemia and elevated plasma LDH levels were found in patients severely affected by aggressive T lymphomas (TCD45⁺). Hypercalcemia is a metabolic disorder frequently associated with advanced malignant disease and was found only in patients with phenotype T (Ruslander *et al.*, 1997; Marconato *et al.*, 2011; Aresu *et al.*, 2015; Harris *et al.*, 2020). Hypercalcemia is due to the production of a peptide (parathyroid hormone related peptide) by neoplastic CD4 T lymphocytes, related to parathyroid hormone (Bryan, 2016; Goldner, 2016). On the other hand, increased LDH levels could correspond to a "shift" toward anaerobic metabolism (increased anaerobic glycolysis), necessary to maintain rapid tumor growth (Fantin *et al.*, 2006). In this regard, previous work found a positive correlation between LDH and the severity of anemia in patients with T-cell lymphoma (Sánchez-Solé, 2019). In this work, the highest values of this enzyme were observed in B-cell lymphomas; however, unlike reported by other authors (Marconato *et al.*, 2013; Marconato *et al.*, 2019), they

were not associated with lower survival. Interestingly, in the present work, we found a direct relationship between high LDH values (greater than 500 IU/l) and lower survival in aggressive CD45$^+$ T lymphomas. To the author's knowledge, this is the first time this relationship has been reported.

Conclusion

Survival time and hematologic and biochemical patterns differed among the immunophenotypes of canine lymphomas. Patients of LTCD45$^-$ phenotype showed higher lymphocyte counts and gamma globulin levels and more prolonged survival. Further study with a more significant number of LTCD45$^-$ dogs is necessary to investigate the role of CD45 molecule in lymphoma pathogenesis, development of paraneoplastic syndromes, and prognosis. Serum LDH activity may provide additional prognostic information in CD45$^+$ T-cell lymphoma.

Conflict of interest

The authors declare that there is no conflict of interest.

Authors' contribution

Rosina Sanchez conducted the research trials, wrote the article, and analyzed the data. Florencia Mosquillo assisted in the methodological development of the flow cytometry. Paulo Jark corrected the final version of the manuscript. Martín Breijo and Paula Pessina designed and supervised the research trials, and assisted in writing and editing the article.

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