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ORIGINAL ARTICLE

Prospective evaluation of flow cytometric characteristics, histopathologic diagnosis and clinical outcome in dogs with naïve B-cell lymphoma treated with a 19-week CHOP protocol

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Abstract

Canine B-cell lymphoma is a clinically heterogenous disease; however, it is generally treated as a single disease entity. The purpose of this clinical trial was to prospectively evaluate naïve canine B-cell lymphoma patients using histopathology, flow cytometry (FC) and a standardized chemotherapy protocol to better define subsets of this disease that may respond differently to treatment. Sixty-four dogs with naïve multicentric B-cell lymphoma were treated with a standardized 19-week CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy protocol. Most of the dogs (84.3%) were diagnosed with diffuse large B-cell lymphoma (DLBCL), followed by nodal marginal zone (7.8%), small B-cell (4.7%), Burkitt-like (1.6%) and follicular lymphoma (1.6%). FC confirmed the diagnosis of B-cell lymphoma in all cases. There were no clear phenotyping differences between the subtypes of B-cell lymphoma detectable by our FC panel. The histologic subtypes in this study exhibited a range of forward scatter values on flow cytometry, but all of the DLBCL cases were higher than a value of 469, while the only cases with a lower forward scatter value were follicular lymphoma and diffuse small B-cell lymphoma. Dogs with DLBCL had a significantly better objective response rate to the CHOP protocol (96.3%) than the non-DLBCL subtypes (70%, $P = .024$). The median progression-free survival time for patients with DLBCL (233 days) was significantly longer than that of all other histopathologic subgroups combined (163 days, $P = .0005$).

KEYWORDS

CHOP, diffuse large B-cell lymphoma, flow cytometry, histopathology, marginal zone lymphoma

1 | INTRODUCTION

Canine lymphoma is a common hematologic malignancy, with a reported annual incidence up to 24 per 100 000 dogs at risk.[1] Diffuse large B-cell lymphoma (DLBCL) is the most common histopathologic subtype of lymphoma in the dog and resembles the DLBCL subtype of non-Hodgkin's lymphoma in humans.[2-4] In both species, DLBCL is characterized by an aggressive disease course with a variable outcome. Canine DLBCL is initially highly responsive to standard chemotherapy protocols; however, drug resistance occurs in most cases

leading to relapse of disease. In humans, gene expression profiling studies have demonstrated that DLBCL can be divided into prognostically important subtypes, including germinal centre B-cell-like (GCB) and activated B-cell-like (ABC).[5-8] When treated with a standard multiagent chemotherapy protocol, patients with GCB-DLBCL have better survival rates, and both subgroups of DLBCL patients treated with rituximab plus CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy see a survival advantage.[5,6] Recent studies have also stratified canine B-cell lymphoma patients by gene expression profiling, demonstrating that two subtypes resembling GCB- and ABC-DLBCL can be distinguished, with the latter demonstrating a poorer outcome.[9,10] However, the distinction of B-cell lymphoma subtypes in dogs has not translated into the clinic as it has in humans.

In contrast, lymphoma in dogs is still commonly treated as a single disease entity. The gold standard treatment for high-grade lymphomas in dogs consists of doxorubicin-based, multiagent chemotherapy protocols such as CHOP. While these protocols are considered the most effective for canine lymphoma, it is not considered a curable disease in dogs, and little progress has been made in the last two decades to improve remission duration and survival.[11] Several systems have been proposed to classify canine lymphoma[4,12]; the World Health Organization (WHO) classification system is most commonly used.[12] However, it is not common in veterinary medicine to pursue a histologic diagnosis to obtain lymphoma subtype, because of time, finances and owners' reluctance in pursuing the more invasive diagnostic test of lymph node (LN) biopsy when the results may not alter treatment course or potential outcome.

Currently, the diagnosis of lymphoma in dogs is routinely made by fine-needle aspiration (FNA) for cytologic assessment, often in combination with flow cytometry (FC) to provide subclassification and prognostic information. While this standard approach is regarded as easy, inexpensive and accurate for obtaining a diagnosis of lymphoma with immunophenotype, it does not allow distinction of histopathologic subtypes of lymphoma, which requires details of nodal architecture obtained through LN biopsy. In contrast, histopathology with gene expression profiling is the basis for human histologic classification, and ultimately directs the course of treatment.

FC is used routinely in both human and veterinary medicine to determine immunophenotype of lymphomas and leukaemias and to subclassify these diseases.[13,14] FC results provide objective data, such as cell size and antigen expression, which can be used to further subclassify lymphomas into clinically relevant subcategories. Of particularly valuable use in veterinary medicine, FC has been useful in distinguishing two common subtypes of T-cell lymphoma, peripheral T-cell and T-zone lymphoma. FC information is often sufficient to guide the treatment plan for canine patients with T-cell lymphoma, in which peripheral T-cell lymphoma requires aggressive treatment and carries a poor prognosis, while T-zone lymphoma typically follows an indolent disease course which often requires only monitoring.[15] FC may also provide prognostic information in B-cell lymphoma. Prior studies have shown that cell surface phenotype, including class II major histocompatibility complex (MHC), CD20 and CD25 expression on B cells, is predictive of prognosis in humans with B-cell lymphoma.[16-18] In addition, large cell size by FC and low-class II MHC expression have been correlated with worse prognosis in dogs with B-cell lymphoma.[19] However, FC cannot accurately distinguish between any B-cell subtypes of lymphoma in veterinary medicine at this time.

In this study, we carried out a prospective clinical trial in naïve canine B-cell lymphoma patients using histopathology, FC and a standardized chemotherapeutic protocol with standardized follow-up to better define subsets of disease that may respond differently to treatment. Few studies are available assessing canine B-cell lymphoma subtype and outcome,[3,20-25] and to our knowledge, no studies have aimed to compare histologic and FC diagnosis in canine lymphoma. It was hypothesized that histologically distinct subclassifications of canine lymphoma would have unique cell surface markers as determined by an FC assay panel, and these subcategories of disease would have variable outcomes when treated with a standardized chemotherapy protocol. A secondary goal of this study was to identify factors associated with patient outcome.

2 | MATERIALS AND METHODS

2.1 | Study population

Dogs diagnosed with multicentric lymphoma whose owners elected treatment with the CHOP protocol were prospectively enrolled in the study from September 2013 through October 2015. There were six study sites encompassing the Front Range Oncology Group (FROG) network, which included the Colorado State University Flint Animal Cancer Center (FACC) and five specialty clinics in the greater Denver metropolitan area. Dogs were eligible for inclusion in this study if they had a cytologic and histologic diagnosis of multicentric B-cell lymphoma with FC information available, lack of previous therapy including prednisone or any chemotherapy agent, and intent to treat with a 19-week CHOP protocol at a FROG network clinic. Breed, sex, weight, age at diagnosis, cytologic diagnosis, immunophenotype and other FC characteristics, histologic diagnosis, and initial complete blood count (CBC) and serum biochemistry parameters were recorded for each dog. Absolute cell counts were recorded and interpreted according to the submitting laboratory's normal reference range. Approximate stage and substage were required for each patient, and these designations were determined by the attending clinician based on the WHO clinical staging system.[26] Information regarding the treatment protocol including adverse events (AEs), dose reductions, dose delays and time to protocol completion was also recorded. The study protocol was approved by the Colorado State University Institutional Animal Care and Use Committee.

2.2 | Flow cytometry

Multiple fine-needle aspirates from a representative LN were performed prior to treatment, and samples were stored in phosphate-

buffered saline with 10% fetal bovine serum and assessed within 72 hours. Samples were shipped directly to the Colorado State University Clinical Immunology Laboratory (Fort Collins, Colorado). FC was carried out as previously described.[15] Samples were analysed with the antibody combinations listed in Table 1 using a 3-laser Coulter Gallios flow cytometer (Beckman Coulter, Brea, California). All FC data analysis was carried out using Kaluza Analysis Software (Beckman Coulter, Brea, California). FC data evaluated in this study included cell size, class II MHC expression, CD21, CD22, CD5, CD45 and CD25 on the neoplastic B cells, and percent CD4 infiltration. Cell size classification was based on forward light scatter of CD21 gated cells measured on a linear scale. Cells from cases of B-cell lymphoma in this study were larger than peripheral blood lymphocytes analysed on the same day (generally from a different patient), and larger than CD5+ T-cells in the same LN. As previously described,[19] cases in which CD21+ lymphocytes had a median forward scatter >720 U were assigned to cell size category "large," whereas the remaining cases were categorized as "medium." It is important to note that lymphocyte size determination will be variable across different flow cytometers and diagnostic laboratories. In order to translate this size value to other flow cytometers, cells classified as "large" had a median forward scatter value >1.6× the value of the CD5+ T-cells detected in the same LN and the same staining tube.[19] The level of class II MHC expression on B cells was determined by the median fluorescence intensity (MFI) of staining on gated B cells as described previously.[19] Samples considered class II MHC "low" were those with an MFI falling in the lowest 15th percentile of all class II expression on B-cell lymphomas. Samples with class II MHC falling between the 15th and 25th percentile in MFI were considered a grey area and not included in the univariate analysis. The level of CD25 expression was measured as the percentage of B cells expressing CD25. Because anti-CD25 was not included in the same staining tube as anti-CD21, the percentage of B cell expressing CD25 was calculated from tube 2 (Table 1) after gating out all cells expressing any T-cell antigens, neutrophils (using CD4 expression), and placing a size gate around the population most consistent with the neoplastic B cells. FC was interpreted prior to knowledge of the histopathology and immunohistochemistry (IHC) results.

TABLE 1 Antibody panels used for immunophenotyping

Tube	Antibody specificity and fluorochrome
Panel (multicolor)	
1	M[a] IgG1-FITC/M IgG1-PE/M IgG1-Alexa 647/M IgG1-Alexa 700/M IgG1-PE-Alexa 750/M IgG1-Pacific Blue
2	CD3-FITC/CD25-PE/CD5-APC/CD8-Alexa 700/CD4-Pacific Blue
3	Class II MHC-FITC/CD22-PE/CD21-Alexa 647
4	Class II MHC-FITC/CD34-PE/CD5-APC/CD14-PE-Alexa 750
5	Class II MHC-FITC/CD18-PE/CD5-APC/CD14-PE-Alexa 750/CD4-Pacific Blue
6	CD5-FITC/CD45-PE/CD21-Alexa 647

Note: Unless otherwise noted, all antibodies were purchased from AbD Serotec, Raleigh, North Carolina. Clones are as follows: CD45 = YKIX716.13, CD18 = YFC118.3 (human CD18), CD4 = YKIX302.9, CD8 = YCATE55.9, CD5 = YKIX322.3, CD21 = CA2.1D6, CD22 = RFB4 (human CD22, purchased from AbCam, Cambridge, Massachusetts), CD3 = CA17.2A12, CD14 = TUK4, class II MHC = YKIX334.2, CD34 = 1H6, CD25 = P2A10 (purchased from eBiosciences, San Diego, California).
[a]M, mouse.

2.3 | Histopathology and immunohistochemistry

All patients had LN biopsies and blood collection prior to treatment, and again at disease progression. LNs were biopsied using a wedge or 8 mm punch technique. A minimum of two biopsies were taken, one placed in formalin for histologic evaluation and the other cut into two pieces and placed in non-formalin preservative (RNAlater, Thermo Scientific, Rockford, Illinois). Samples collected at FROG study sites were shipped overnight to the FACC for routing, processing and storage. Formalin-fixed samples were processed and paraffin embedded. An H&E slide was made, and IHC performed for immunophenotyping consisting of CD3 and Pax5. Five-micron thick sections from formalin-fixed, paraffin-embedded tissues were cut and immunostained with CD3 (T cells, clone LN10; Leica Biosystems, Newcastle Upon Tyne, UK) and Pax5 (B cells, clone DAK-Pax5; Dako, Carpintaria, California). Deparaffinization, antigen retrieval, IHC staining and counterstaining were performed on the BOND-MAX automated staining system using the Bond Polymer Detection system (Leica Biosystems, Newcastle Upon Tyne, UK). Samples were initially reviewed by a single independent board-certified anatomic pathologist (E.J.E.) who made a diagnosis and subclassification according to the WHO criteria for malignant canine lymphoma.[12] Results were reported to the study site and the FACC. Histopathology was subsequently reviewed by a second independent board-certified anatomic pathologist (K.L.H.) utilizing the same criteria.[12] Any samples in disagreement with the initial diagnosis were reviewed by both pathologists to reach a consensus.

2.4 | Nineteen-week CHOP protocol and response criteria

All dogs were treated with an identical multiagent chemotherapy protocol as outlined in Table 2. All dogs had a CBC performed at each chemotherapy visit. A CBC was also performed 1 week after the first doxorubicin treatment (week 5). Dose delays and reductions were performed because of AEs at the discretion of the attending clinician, and general guidelines were provided. Response to therapy was determined by using the Veterinary Cooperative Oncology Group (VCOG) v1.0 response evaluation criteria for lymphoma.[27] The objective response rate (ORR) was defined as the sum of patients with a

TABLE 2 Nineteen-week CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) protocol dosing schedule

	Week															
	1	2	3	4	6	7	8	9	11	12	13	14	16	17	18	19
Vincristine 0.7 mg m^{-2} IV	•		•		•		•		•		•		•		•	
Cyclophosphamide 200-250 mg m^{-2} PO[a]		•				•				•				•		
Doxorubicin 30 mg m^{-2} IV[b]				•				•				•				•
Prednisone (mg kg^{-1} per day) PO	2.0	1.5	1.0	0.5												

[a]Cyclophosphamide was administered on a single day with furosemide 1 mg kg^{-1} PO.
[b]Patients with body weight <15 kg received doxorubicin at 1 mg kg^{-1}.

TABLE 3 Baseline characteristics of the patient population

Parameter		All	DLBCL	nMZL	SBCL	BL	FL
n		64	54	5	3	1	1
Age (years)	Median (range)	9.0 (3.0-13.8)	8.9 (3.0-12.3)	8.0 (6.2-13.8)	9.1 (7.0-13.1)	10.5	9.3
Sex	Neutered male	42 (65%)	34 (64%)	4 (80%)	3 (100%)	1	
	Male	1 (2%)	1 (2%)				
	Spayed female	21 (33%)	18 (34%)	1 (20%)			1
Weight (kg)	Median (range)	29.6 (5.5-52.7)	28.7 (5.5-52.7)	37.7 (6.3-45.3)	31.6 (28.6-39.1)	33.5	29.8
Breed	Mixed breed	15 (23%)	12 (22%)	1 (20%)	2 (67%)		
	Golden Retriever	7 (11%)	6 (11%)	1 (20%)			
	Labrador Retriever	7 (11%)	5 (9%)		1 (33%)	1	
	Bernese Mountain Dog	4 (6%)	4 (7%)				
	German Shepherd	4 (6%)	4 (7%)				
	Boxer	3 (5%)	2 (4%)				1
	Other (≤ 2 each)	24 (38%)	21 (39%)	3 (60%)			
Stage	II	1 (2%)	1 (2%)				
	III	35 (54%)	30 (56%)	3 (60%)	1 (33%)		1
	IV	23 (36%)	19 (35%)	2 (40%)	1 (33%)	1	
	V	5 (8%)	4 (7%)		1 (33%)		
Substage	a	58 (91%)	50 (93%)	5 (100%)	2 (67%)		1
	b	6 (9%)	4 (7%)		1 (33%)	1	
Cell size[a]	Large	2 (3%)	1 (2%)	1 (20%)			
	Medium	62 (97%)	53 (98%)	4 (80%)	3 (100%)	1	1
MHC II[b]	High	40 (62%)	34 (63%)	3 (60%)	2 (67%)		1
	Low	19 (30%)	16 (30%)	1 (20%)	1 (33%)	1	
	NA	5 (8%)	4 (7%)	1 (20%)			
Calcium status	Normal	63 (98%)	54 (100%)	4 (80%)	3 (100%)	1	1
	Elevated	1 (2%)		1 (20%)			
Anaemia	No	54 (84%)	47 (87%)	3 (60%)	2 (67%)	1	1
	Yes	10 (16%)	7 (13%)	2 (40%)	1 (33%)		
Lymphocytosis	No	58 (91%)	48 (89%)	5 (100%)	3 (100%)	1	1
	Yes	6 (9%)	6 (11%)				
Thrombocytopenia	No	54 (84%)	44 (81%)	5 (100%)	3 (100%)	1	1
	Yes	10 (16%)	10 (19%)				

Abbreviations: BL, Burkitt-like lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; nMZL, nodal marginal zone lymphoma, SBCL, small B-cell lymphoma.
[a]Cell size determined by flow cytometry.
[b]Class II MHC expression determined by flow cytometry.

TABLE 4 Descriptive information and outcome for all dogs with B-cell lymphoma (n = 64) treated with a standardized 19-week CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy protocol

Histologic diagnosis	n (Overall frequency)	MPFS (days)	MST (days)
All B-cell lymphoma	64	225	297
Diffuse large B-cell lymphoma	54 (84.3%)	233	325
Nodal marginal zone lymphoma	5 (7.8%)	24	26
Small B-cell lymphoma	3 (4.7%)	146.5	114
Burkitt-like lymphoma	1 (1.6%)	163	163
Follicular lymphoma	1 (1.6%)	Undefined	Undefined

Abbreviations: MPFS, median progression-free survival; MST, median overall survival time.

complete response (CR) or partial response (PR). Progression-free survival (PFS) time was defined as the time from initiation of the 19-week CHOP protocol to disease progression or death from any cause. Overall survival time (OST) was defined as the time from CHOP initiation to death from any cause. All AEs were prospectively recorded by the attending clinician and graded based on the VCOG common terminology criteria v1.1.[28]

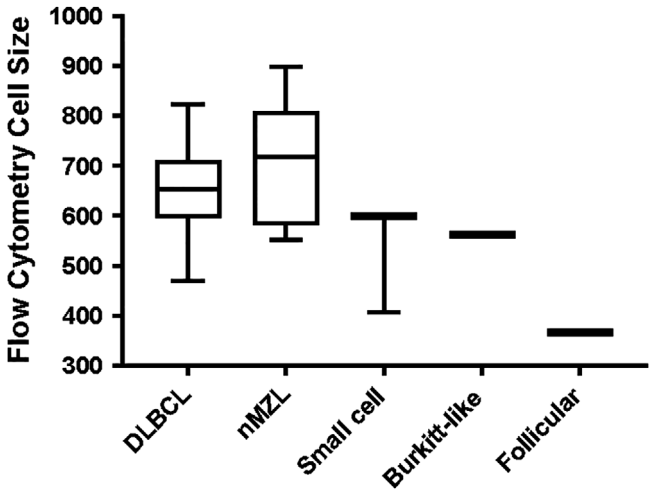

FIGURE 1 Box and whisker plot comparing flow cytometry cell size measured by median forward light scatter for all subgroups of B-cell lymphoma, including DLBCL, diffuse large B-cell lymphoma (n = 54), nMZL, nodal marginal zone lymphoma (n = 5), small B-cell lymphoma (n = 3), Burkitt-like lymphoma (n = 1), and follicular lymphoma (n = 1). Statistically significant differences were not identified between the groups; however, all patients diagnosed with DLBCL, nMZL and Burkitt-like lymphoma fell above a minimum forward light scatter value of approximately 469. The whiskers are set at the minimum and maximum value

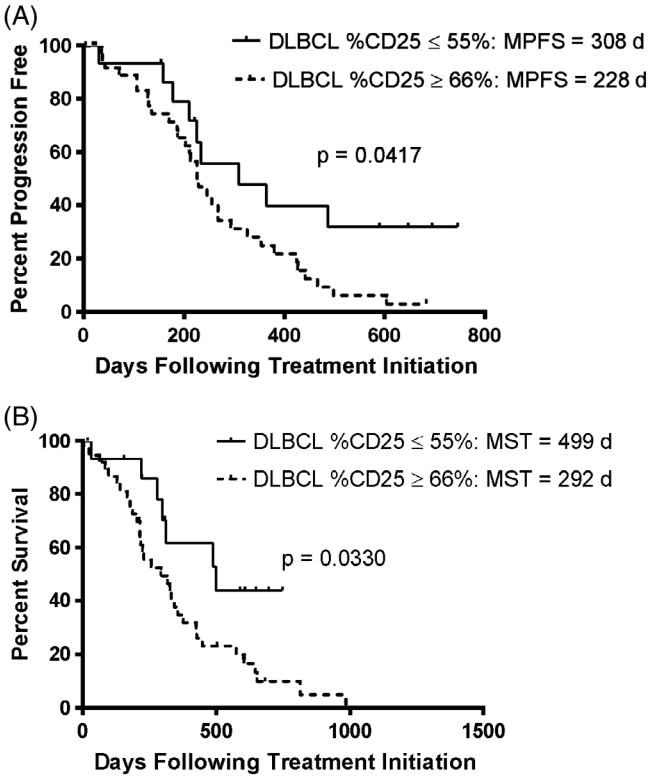

FIGURE 2 Kaplan-Meier curves depicting progression-free (A) and overall survival time (B) of diffuse large B-cell lymphoma (DLBCL) patients with a higher percent of CD25+ B-cells (≥66%) compared to DLBCL patients with a lower percent of CD25+ B-cells (≤55%) receiving CHOP chemotherapy. *P*-values represent univariate log-rank values

2.5 | Statistical analysis

Continuous data were expressed as median and range, and categorical data as frequencies and percentages. The PFS and OST were calculated from the date of treatment initiation to the date of progressive disease and death, respectively. Dogs that died were considered to be dead of either their disease or secondary to complications of treatment. Dogs that were lost to follow up were considered to have died of their disease if they were known to be out of remission at their last visit. Dogs were censored if they had not developed progressive disease (PD) at the time of data analysis, or if they were withdrawn or lost to follow up before PD development. Categorical variables were compared between cohorts using a two-tailed Fisher's exact test. The Kaplan-Meier method was used to estimate and display the distribution of median PFS (MPFS) and median OST (MST). Clinical data at the time of presentation, including signalment, approximate stage, substage, body weight, absolute monocyte count, absolute neutrophil count, hypercalcemia, lymphocytosis, anaemia, thrombocytopenia, whether the CHOP protocol was completed, flow cytometric data listed above, and the presence of treatment delays, dose reductions, or AEs were evaluated for the effect on PFS and OST. Differences between groups were compared using both log-rank and Gehan-Breslow-Wilcoxon analysis. CD25 expression cut-offs for MPFS and

TABLE 5 Adverse events

Total adverse events (n = 267)		Grade 1	2	3	4
Gastrointestinal	Hyporexia	19	14	8	
	Diarrhoea	27	9	3	
	Nausea	2			1
	Vomiting	17	7	1	
Hematologic	Anaemia	20	3		
	Neutropenia	23	16	6	5
	Thrombocytopenia	1	1		
Constitutional	Fever	2		2	
	Lethargy	14	3	5	
	Pelvic limb weakness	3			
	Weight loss	5	2	1	
Metabolic	Albumin decrease	2			
	ALP elevation	5	4	4	1
	ALT elevation	2	1	2	
	Creatinine elevation	1			
	Total bilirubin elevation	1	1		
Urinary	Cystitis	2			
	Haematuria	1		1	
	Proteinuria	5		1	
	Stranguria			1	
Respiratory	Coughing	2			
	Dyspnoea	1			
	Pleural effusion			1	
Cutaneous	Alopecia/haircoat changes	5			
	Dermatitis		1		
	Otitis externa	1	1		

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase.

MST were reached by first comparing CD25 expression above and below the median, and then evaluating natural breakpoints with increasing CD25 until the highest CD25 that showed a significant difference between the two groups was identified. Multivariate analysis was performed using forward and reverse stepwise Cox regression, incorporating variables reaching significance on univariate analysis. Variables with values of $P \leq .05$ were considered significant. All clinical statistical analysis was performed with commercial software packages (Prism v.7.0, GraphPad Software, La Jolla, California; SPSS v.21, IBM, Armonk, New York).

3 | RESULTS

3.1 | Patient population

A total of 93 dogs with multicentric lymphadenopathy were cytologically diagnosed with lymphoma by a board-certified clinical pathologist. Nineteen patients were diagnosed with T-cell lymphoma via FC and/or histopathology and were therefore not included in this study. A total of 74 dogs were diagnosed with B-cell lymphoma. Of those, 64 dogs had both FC and histopathology available, satisfied inclusion criteria, and were enrolled in the study. Information regarding signalment, body weight, approximate stage, substage, calcium status, presence of anaemia, lymphocytosis, thrombocytopenia and FC characteristics including cell size and class II MHC expression is presented in Table 3. Full staging was not performed in all dogs; however, every patient was assigned an approximate stage and substage by the attending clinician. Twenty-three dogs (36%) had thoracic radiographs, 22 (34%) received an abdominal ultrasound, and none had bone marrow sampling performed.

All 64 dogs started the CHOP protocol, with 37 dogs (58%) completing the protocol. Of the 27 dogs that did not complete the CHOP protocol, 18 developed progressive disease during the course of treatment, five developed unrelated conditions, three discontinued therapy at the owner's request, and one developed a second tumour type. Four dogs (6.3%) were alive at the time of data analysis. Forty-six dogs (71.8%) died from lymphoma, seven (10.9%) died of other

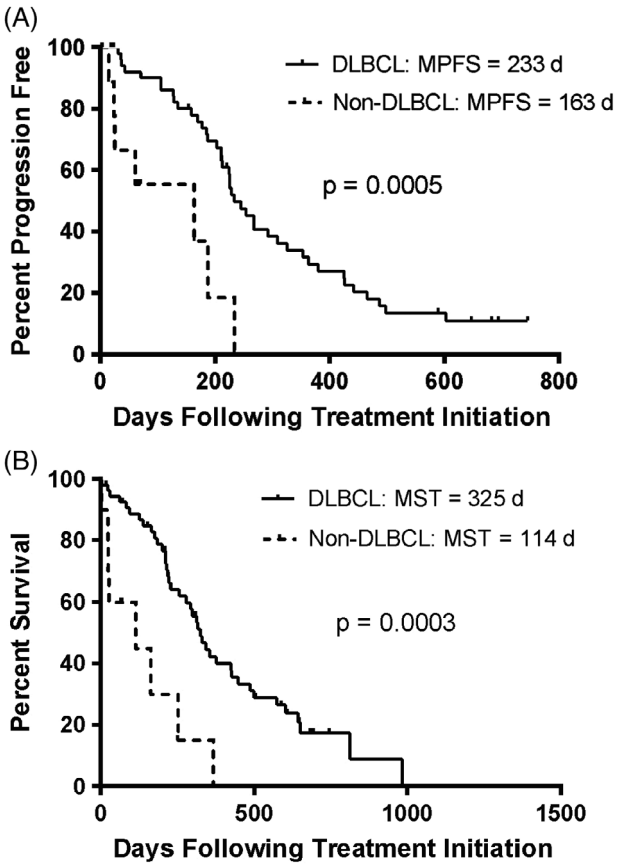

FIGURE 3 Kaplan-Meier curves depicting progression-free (A) and overall survival time (B) of patients with diffuse large B-cell lymphoma (DLBCL) compared to other subtypes of B-cell lymphoma (non-DLBCL) receiving CHOP chemotherapy. *P*-values represent univariate log rank values

causes, four (6.3%) were lost to follow-up, and three (4.7%) were withdrawn. Twenty-three dogs (36%) received rescue therapy at the time of disease progression/relapse. Agents/protocols employed at the time of relapse included L-asparaginase/lomustine (6 dogs), L-asparaginase (5 dogs), CHOP (3 dogs), LOPP (3 dogs), doxorubicin (2 dogs), CHOP/radiation (1 dog), lomustine (1 dog), Tanovea (1 dog), and EZN-3042 clinical trial (1 dog).

3.2 | Histopathology

Histopathology was performed in all dogs; frequencies along with outcome data are listed in Table 4.

3.3 | Flow cytometry

FC was performed in all 64 dogs and confirmed the diagnosis of B-cell lymphoma in all cases. There were insufficient numbers of DLBCL, nMZL, small B-cell, Burkitt-like or follicular lymphoma to assess immunophenotyping differences between these groups detected by the FC panel used in this study. Cell size as determined by FC was compared for all subtypes of B-cell lymphoma. Statistically significant differences were not identified between the groups; however, all patients diagnosed with DLBCL, nMZL and Burkitt-like lymphoma fell above a minimum forward light scatter value of approximately 469 as established by FC (Figure 1). All B-cell lymphoma patients expressed class II MHC at varying levels. As illustrated in Figure 2, DLBCL patients with a higher percent of CD25+ B-cells (≥66%) had a statistically significant decrease in PFS and OST compared to dogs with a lower percent of CD25+ B-cells (≤55%). The median value for percentage of CD25+ B-cells was 88.5 (range 1-99). Median PFS for DLBCL patients with ≤55% CD25 expression (n = 16) was 308 days (range 18-745), and for DLBCL patients with ≥66% CD25 expression (n = 38) was 228 days (range 3-682); HR = 0.48[0.25-0.90]; *P* = .0417. Median OST for DLBCL patients with ≤55% CD25 expression (n = 16) was 499 days (range 18-745), and for DLBCL patients with ≥66% CD25 expression (n = 38) was 292 days (range 3-984); HR = 0.43[0.22-0.83]; *P* = .0330.

TABLE 6 Factors identified by univariate analysis to be prognostic for progression-free survival for dogs with DLBCL (n = 54)

Factor		N	Median PFS	Log-rank *P*	GBW *P*	Log-rank HR (95% CI)
Age	<5.5 y	8	212	.0167	.0807	2.53 (0.79-8.08)
	≥5.5 y	46	267			
CD25[a]	≤55%	16	308	.0417	.1395	0.48 (0.25–0.90)
	≥66%	38	228			
Completed CHOP	No	21	128	.0067	<.0001	2.37 (1.04-5.41)
	Yes	33	292			
Lymphocytosis	No	48	267	<.0001	<.0001	0.10 (0.0063-0.14)
	Yes	6	42			
Weight	<29 kg	27	225	.0152	.0689	2.04 (1.08-3.86)
	≥29 kg	27	325			

Abbreviations: CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; DLBCL, diffuse large B-cell lymphoma; GBW, Gehan-Breslow-Wilcoxon; HR (95% CI), hazard ratio (95% confidence interval); PFS, progression-free survival.
[a]CD25 expression on B cells as determined by flow cytometry.

TABLE 7 Factors identified by univariate analysis to be prognostic for overall survival for dogs with DLBCL (n = 54)

Factor		N	Median OST	Log-rank *P*	GBW *P*	Log-rank HR (95% CI)
Age	<5.5 y	8	207	.0004	.0023	3.55 (1.02-12.4)
	≥5.5 y	46	375			
CD25[a]	≤55%	16	499	.0330	.0482	0.43 (0.22-0.83)
	≥66%	38	292			
Completed CHOP	No	21	176	<.0001	<.0001	3.54 (1.47-8.53)
	Yes	33	426			
Lymphocytosis	No	48	355	<.0001	<.0001	0.18 (0.029-1.08)
	Yes	6	112			

Abbreviations: CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; DLBCL, diffuse large B-cell lymphoma; GBW, Gehan-Breslow-Wilcoxon; HR (95% CI), hazard ratio (95% confidence interval); OST, overall survival time.
[a]CD25 expression on B cells as determined by flow cytometry.

TABLE 8 Factors significant for progression-free survival or overall survival time for dogs with DLBCL (n = 54) on multivariate analysis

	PFS		OST	
Factor	*P*	HR (95% CI)	*P*	HR (95% CI)
Age	.007	3.75 (1.43-9.80)	.005	3.98 (1.52-10.5)
CD25[a]			.049	0.42 (0.18-0.996)
Completed CHOP	.027	2.43 (1.11-5.34)	.003	3.34 (1.53-7.33)
Lymphocytosis	<.001	0.068 (0.018-0.27)	<.001	0.109 (0.035-0.337)
Body weight	.011	2.61 (1.25-5.46)		

Abbreviations: CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; DLBCL, diffuse large B-cell lymphoma; HR (95% CI), hazard ratio (95% confidence interval); PFS, progression-free survival; OST, overall survival time.
[a]CD25 expression on B cells as determined by flow cytometry.

3.4 | Adverse events

There was a total of 267 documented AEs in the study which are listed in Table 5. Forty-nine dogs (77%) experienced AEs during treatment. Forty percent of AEs were in the gastrointestinal category, followed by hematologic with 28%. Neutropenia was the single most common reported AE (19% of all reported AEs), in which grades 1 and 2 predominated. Nine dogs (14%) in this study were hospitalized secondary to chemotherapy-induced toxicity, primarily after receiving cyclophosphamide (4 dogs). Approximately one-third of dogs required dose reductions on this protocol, in which the most common chemotherapy agent reduced was vincristine. Two patients received vinblastine in a modified CHOP protocol, which was substituted for vincristine due to AEs. Thirty-nine percent of dogs experienced treatment delays during the protocol.

3.5 | Patient outcomes

For all dogs with B-cell lymphoma, the objective response rate (ORR) to the 19-week CHOP chemotherapy protocol was 92.2%. Fifty-five dogs (85.9%) experienced a CR, four (6.3%) achieved a PR, three (4.7%) had stable disease and two (3.1%) had PD as their best response to therapy. For dogs with DLBCL, the ORR was significantly higher (96.3%) compared to dogs with non-DLBCL (70%, *P* = .024). For the non-DLBCL dogs, the ORR for nMZL and small cell B-cell lymphoma was 60% and 67%, respectively. Each dog with Burkitt-like and follicular lymphoma achieved a CR. Sixteen of the 64 dogs were censored from survival analysis. Dogs that were alive at the time of data analysis or withdrawn due to owner's wishes or lymphoma-unrelated deaths were censored. The median follow-up time in censored patients was 682 days (range 18-745). The MPFS for all dogs with B-cell lymphoma in this study was 225 days (range 2-745), and the MST was 297 days (range 2-984). One dog died 2 days after the start of chemotherapy following treatment with vincristine.

For dogs with DLBCL, the MPFS and MST were 233 days (range 3-745) and 325 days (range 3-984), respectively. Both PFS and OST of dogs with DLBCL were significantly longer than that of all other histopathologic subgroups combined (163 days, HR = 0.27 [0.069-1.07]; *P* = .0005; and 114 days, HR = 0.28[0.078-0.98]; *P* = .0003), as illustrated in Figure 3. Prognostic factors identified on univariate analysis as significant for PFS for dogs with DLBCL included age, CD25 expression, completion of CHOP, presence of lymphocytosis and body weight (Table 6). On multivariate analysis, age, completion of CHOP, lymphocytosis and body weight remained significant for PFS (Table 8). Prognostic factors identified on univariate analysis as significant for overall survival for dogs with DLBCL included age, percentage of CD25 expression, completion of CHOP

and presence of lymphocytosis (Table 7). On multivariate analysis, all of these factors remained significant for OST (Table 8).

4 | DISCUSSION

Dogs diagnosed with B-cell lymphoma without a histologic diagnosis are routinely started on a CHOP-based chemotherapy protocol as standard-of-care therapy, and response to therapy is then monitored. While this approach may seem logical, we recognize clinically that dogs with different subtypes of B-cell lymphoma may respond differently to this therapy, which may prove costly for clients and ultimately compromise patient outcome in the long run. Hence, utilizing FC as a less invasive diagnostic tool to fill this role in veterinary medicine is particularly attractive for the patient, client, and clinician.

Results of this prospective study found that FC confirmed the diagnosis of B-cell lymphoma in all cases; however, there were no clear phenotyping differences between different subtypes of B-cell lymphoma detectable by the antibodies used in this study. We feel that the scope of this study was ultimately limited by the small number of antibodies that detect canine B cells and a small study size. Including additional patients with FC and histopathologic diagnoses retrospectively may provide the power to find differences for further stratification of FC parameters. LN biopsy remains the gold standard diagnostic tool to differentiate between the most common subtypes of canine B-cell lymphoma; DLBCL and non-DLBCL subgroups.

The histologic subtypes in this study exhibited a range of forward scatter values on FC, but all of the DLBCL cases were higher than a value of 469, while the only cases with a lower forward scatter value were follicular lymphoma and diffuse small B-cell lymphoma. Cell size greater than 469 does not rule out subtypes other than DLBCL, but cell size smaller than 469 can be used as a first step in identifying the less common forms of B-cell lymphoma. Unpublished observations from our laboratory indicate that approximately 15% of all nodal B-cell lymphomas (n = 7749) are in this group. Further work is under way to better characterize FC parameters of less common non-DLBCL variants of B-cell lymphoma in canine patients, but currently, as noted above, only histopathology can distinguish these various subtypes. It is important to note that the histologic criteria defining cell size depend on comparison of nuclear size to a standard cell in the tissue section (typically red blood cells). FC defines cell size based on an objective assessment of the median size of the entire volume of the cell in suspension. Therefore, descriptions of cells as being "small, medium and large" will differ between the classification systems.

It is now recognized that the majority of cases of canine B-cell lymphoma analysed by FC are composed of medium-sized CD21 cells with variable, although generally high, MHC class II expression.[14] This description is consistent with the findings of the present study. In addition, a recent study evaluating the FC findings from 37 histologically confirmed cases of DLBCL found a similar consistent phenotype.[21] In prior studies evaluating FC parameters of canine B-cell lymphoma, patients were divided into "medium" and "large" cell size, and particularly large cell types had a significantly poorer outcome.[19] Only two patients in the present study fell into the "large" cell size category, and cell size was not correlated with survival in DLBCL. Rao et al also previously reported that low class II MHC levels in dogs with B-cell lymphoma was associated with poor survival,[19] a finding also noted by Pinheiro.[29] In the current study, class II MHC expression was not correlated with outcome in DLBCL. One possible explanation for the discrepancy in these findings may be that within some histopathologic subtypes, cell size and class II MHC levels are not important for survival. It should also be noted that in the two prior studies, no attempt was made to subclassify the B-cell lymphomas by histologic subtype.

In this study, dogs with DLBCL who had ≥66% CD25 expression on B cells had a worse prognosis. While CD25 expression on B cells has been previously evaluated in canine B-cell chronic lymphocytic leukaemia,[30] it has not been extensively evaluated in canine DLBCL. CD25 comprises the alpha chain of the IL-2 receptor, which binds the growth factor IL-2 and stimulates the clonal expansion and maturation of activated T- or B-lymphocytes.[31] Human DLBCL patients with a higher CD-25 positivity (defined as over 60% CD25 positivity) had a less favourable response and inferior PFS compared to patients with lower CD25-positivity, in which the majority of patients received rituximab and CHOP chemotherapy.[18] In a recent study of dogs with high-grade B-cell lymphoma, the PFS was also significantly shorter in a CD25-high group than that in a CD25-low group.[32] Collectively based on these findings, CD25 may be a promising prognostic marker and potential therapeutic target in DLBCL in both human and canine patients.

While DLBCL was expected to comprise the majority of canine patients with B-cell lymphoma, a goal of this study was to characterize patients with less common non-DLBCL subtypes of lymphoma and to assess their response to CHOP chemotherapy. After DLBCL, the second most common B-cell lymphoma subtype was nMZL comprising 7.8% of the patient population. On FC, the five patients with nMZL exhibited a wide range of cell size; however, most were classified as "medium" size CD21+, with one "large" cell size. Class II MHC expression was also variable in this subset of patients and was collectively higher than class II MHC expression in dogs with DLBCL. The ORR to CHOP chemotherapy for dogs with nMZL was poor at 60%, and only one patient completed the chemotherapy protocol. These factors may have contributed to the poor outcome of these patients. The description of nMZL patients in this study correlates with a recent retrospective study of 35 canine patients with nMZL.[33] In that study, all nMZL dogs had stage V disease mainly composed of medium-sized CD21+ cells. The outcome was also poor when treated with a CHOP-based protocol, with a median time to progression and lymphoma-specific survival time of 149 and 259 days, respectively.

Canine small B-cell lymphoma is generally considered an indolent disease. However, results from this study suggest that there may be subsets of this disease that may have a more aggressive clinical course. This was previously suggested by Ponce et al who identified four cases of small B-cell lymphoma (not otherwise specified) with a high mitotic rate and Ki-67 index suggestive of a high-grade lymphoma phenotype.[34] In that study, histologic grading was determined

by the size of cells on histopathology and by mitotic index (MI). Cases showing a majority of small-sized cells on histopathology, and a low or medium MI were classified as low-grade lymphomas. Cases showing a majority of medium- and large-sized cells on histopathology, and a high MI were classified as high-grade lymphomas. For intermediate, atypical and/or doubtful cases, final tumour subclassification was based on determining Ki-67 index. This study also raised the possibility that a low-grade small B-cell lymphoma may transform to a higher grade. In this scenario, the patient may still have small- to medium-sized cells on histopathology, but a higher MI may be present. Collectively, the findings of these studies suggest that the clinical course of nMZL and small B-cell lymphoma are not always indolent as previously thought, and the best treatment option still needs to be determined. In the scope of this study, we found that there were inadequate sample sizes to fully investigate the less common subtypes of B-cell lymphoma (follicular and Burkitt-like), and further investigation is warranted.

Several factors have been shown to influence the prognosis of dogs with lymphoma, however, few studies have focused specifically on dogs with DLBCL.[20-25] The present study added to our limited knowledge of prognostic factors for dogs with DLBCL. Factors identified on multivariate analysis as significant for MPFS and/or MST included age, body weight, B cell CD25 expression, presence of lymphocytosis and completing CHOP. The lack of consensus of prognostic factors associated with outcome in this handful of studies may be due to the evaluation of a more homogenous group of dogs in the present study. No dogs in this study were pre-treated with steroids and there was a small representative proportion of stage V and substage b patients, likely due to the requirement to meet inclusion criteria for a prospective clinical trial.

While this study was conducted by a clinical trials network, utilizing multiple study sites also proved to be a limitation of this study. For example, there was variation in management of patients due to multiple clinicians and hospital staff. In addition, shipping FC samples from external study sites resulted in the loss of enrollment of at least two patients due to non-viable samples. Strengths of the data collected in this multicentre study include its prospective nature, utilizing two independent pathologists for review of histopathology samples, and standardizing FC analysis in a well-known laboratory.

In conclusion, this is the first prospective study to compare FC characteristics to histologic diagnosis in lymphoma in dogs. Results of this study further demonstrate that canine B-cell lymphoma is a heterogenous disease. Despite the dogma that a B-cell phenotype translates to a better prognostic indicator vs a T-cell phenotype, there are subsets of B-cell lymphoma associated with median survivals that are as poor as those reported for T-cell lymphoma, and which may require a more tailored therapy. Further studies are needed to determine additional factors that may allow for further patient stratification to eventually modify therapy to improve outcome.

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CONFLICT OF INTEREST

D.H.T is a co-editor of this journal.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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