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Clinical outcome and prognostic factors in dogs with B-cell chronic lymphocytic leukemia: A retrospective study

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Abstract

Background: B-cell chronic lymphocytic leukemia (BCLL) in dogs generally is considered an indolent disease, but previous studies indicate a wide range in survival times.

Objectives: We hypothesized that BCLL has a heterogeneous clinical course, similar to chronic lymphocytic leukemia in humans. We aimed to assess presentation and outcome in dogs with BCLL and evaluate the prognostic relevance of clinical and flow cytometric factors.

Animals: One hundred and twenty-one dogs with BCLL diagnosed by flow cytometry. Three breed groups were represented: small breed dogs (n = 55) because of increased risk of BCLL; Boxers (n = 33) because of preferential use of unmutated immunoglobulin genes; and other breeds (n = 33).

Methods: Retrospective study reviewing signalment, clinicopathologic data, physical examination findings, treatment, and survival of dogs with BCLL. Cellular proliferation, determined by the percentage of Ki67-expressing CD21+ B-cells by flow cytometry, was measured in 39 of 121 cases. Clinical and laboratory variables were evaluated for association with survival.

Results: The median survival time (MST) for all cases was 300 days (range, 1-1644 days). Boxers had significantly shorter survival (MST, 178 days) than non-Boxers (MST, 423 days; $P < .0001$), and no significant survival difference was found between small breeds and other non-Boxer breeds. Cases with high Ki67 (>40% Ki67-expressing B-cells) had significantly shorter survival (MST, 173 days) than did cases with <40% Ki67 (MST undetermined; $P = .03$), regardless of breed. Cases with a high lymphocyte count (>60 000 lymphocytes/μL) or clinical signs at presentation had significantly shorter survival.

Conclusions and Clinical Importance: B-cell chronic lymphocytic leukemia had a variable clinical course and Boxer dogs and cases with high Ki67 had more aggressive disease.

Abbreviations: BCLL, B-cell chronic lymphocytic leukemia; CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; CR, complete response; IQR, interquartile range; MHC, major histocompatibility complex; MST, median survival time; MTD, maximum tolerated dose; PARR, PCR for antigen receptor rearrangements; PD, progressive disease; PR, partial response; SDZ, stable disease.

KEYWORDS
canine, flow cytometry, immunophenotyping, Ki67, lymphocytosis, lymphoma

1 | INTRODUCTION

B-cell chronic lymphocytic leukemia (BCLL) is a malignancy of small-sized B-cells in the blood and bone marrow and a common form of leukemia in dogs. It represented 8% of all samples with suspicion of lymphoproliferative disease (of any site) in 1 study[1] and 36% of CLL cases in dogs in another study.[2] Diagnostic criteria for BCLL vary, generally requiring >5000 to 6000 lymphocytes/μL in the blood and identification of B-cell expansion by immunophenotyping.[1,3-6] Some studies incorporate small mature cytomorphology or small cell size by flow cytometry as inclusion criteria, and some exclude cases with moderate-to-severe lymphadenopathy or splenomegaly. The disease generally affects older dogs (median age, 10-11.9 years).[1,3,6] A previous study indicated that certain small dog breeds have increased risk of BCLL, and approximately half of cases have lymphadenopathy or splenomegaly.[1]

In people, chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) is a common B-cell neoplasm in the blood, bone marrow, lymph nodes, and spleen.[7] A diagnosis of CLL requires >5000 B-cells/μL in the blood and SLL requires lymphadenopathy, splenomegaly, or both with <5000 B-cells/μL, but these entities are considered different manifestations of the same disease.[8] In humans, CLL/SLL has a highly variable clinical course and genetic and clinical variables are used to assess prognosis.[7,9,10] In dogs, BCLL generally is considered an indolent disease, but studies indicate a wide range in survival times.[3,11]

The goal of our study was to assess survival and prognostic factors in a larger number of dogs with BCLL, defined by an expansion of small-sized B-cells in the blood by flow cytometry. We did not exclude cases with lymphadenopathy, splenomegaly or cytopenias, because doing so may exclude true BCLL cases. Although it is possible some cases in our study had a different small B-cell neoplasm, we wanted to include all cases with small cell B-cell lymphocytosis in the blood, because it is unknown whether cases with tissue infiltration are a different manifestation of BCLL vs an entirely different neoplasm. We evaluated Ki67 expression for association with survival. It is a marker of proliferation with prognostic value in high-grade B-cell lymphomas of dogs when measured by flow cytometry.[12] In humans with CLL, high Ki67 expression is associated with poorer outcome, whether measured in neoplastic peripheral lymphocytes, lymph node proliferation centers, or plasma.[13-15] Additionally, Boxers with BCLL preferentially rearrange unmutated immunoglobulin heavy variable region genes, which is a poor prognostic indicator in humans with CLL, and our study aimed to examine breed-related differences in presentation and outcome.[16-18] We hypothesized that BCLL in dogs has a variable clinical course similar to CLL in humans and that Boxers and dogs with high Ki67 expression would have more aggressive disease.

2 | MATERIALS AND METHODS

2.1 | Study population

The Colorado State University Clinical Hematopathology laboratory database was queried for BCLL cases in dogs with blood submitted for flow cytometry between October 2010 and October 2018. Inclusion criteria included: (a) >5000 lymphocytes/μL on CBC, (b) >60% of lymphocytes expressed CD21, (c) no cells expressed CD34, (d) B-cells were small-sized, defined by B-cell:neutrophil median forward scatter ≤0.60.[1,11,16] Cases initially were selected randomly to represent the general BCLL population. Additional Boxer cases were sought because Boxers with BCLL preferentially rearrange unmutated immunoglobulin heavy variable region genes, which is associated with poor prognosis in humans with CLL.[16] English bulldogs were excluded because this breed develops a condition of non-neoplastic, polyclonal B-cell expansion.[19] We rarely have identified this polyclonal B-cell syndrome in other breeds, but no cases in this BCLL study had the characteristics of that syndrome.

For controls to evaluate CD21, CD25, and class II major histocompatibility complex (MHC) expression on normal B-cells, routine flow cytometry was performed on blood from 30 healthy dogs with normal lymphocyte subsets by flow cytometry and polyclonal gene rearrangements by PCR for antigen receptor rearrangements (PARR).

Between June 2017 and August 2019, we performed Ki67 staining by flow cytometry (Appendix S1) on randomly selected BCLL cases, nodal B-cell lymphoma cases, and controls. Nodal B-cell lymphoma cases were lymph node samples with a homogeneous expansion of CD21+ B-cells with a median forward scatter >500 by flow cytometry. Previously, B-cell lymphoma cases meeting these criteria were predominantly diffuse large B-cell lymphoma.[20] For size comparison, canine blood neutrophils had a mean forward scatter of 620 (SD, 61). Additionally, Ki67 staining was performed on 7 lymph node aspirates and 5 blood samples from 12 control dogs with no evidence of lymphoproliferative disease. All BCLL cases with Ki67 data obtained between September 2017 and October 2018 were contacted for medical records.

In total, 435 BCLL cases were contacted for the outcome study from 3 queries ([a] initial query of cases of any breed, [b] additional Boxer cases, and [c] BCLL cases of any breed with Ki67 data) and the 121 cases with available medical records were included in the study. Additionally, separate from the outcome study population, a population of additional BCLL cases, nodal B-cell lymphoma cases, and controls had Ki67 analysis performed.

2.2 | Clinical variables

Medical records were reviewed for signalment, physical examination and imaging findings, clinicopathologic data, clinical signs, treatment,

and survival data. Anemia was defined by a hematocrit below the laboratory's reference interval. Thrombocytopenia was defined by a platelet count below the laboratory's reference interval with no evidence of platelet clumping. Hyperglobulinemia was defined by the upper limit of the reference interval on available serum biochemistry panels. Visceral lymph node, spleen, and liver abnormalities, including enlargement or masses, were identified by palpation, ultrasound examination, or thoracic or abdominal radiography. Blood smear evaluations, cytology reports, and histology reports from board-certified pathologists were reviewed. Lymphocyte size was determined by a variety of methods across pathologists, including micrometer measurement, comparison to erythrocyte diameter, or comparison to neutrophil diameter of the lymphocyte nucleus only or the entire lymphocyte diameter.

2.3 | Flow cytometry

Flow cytometry was performed as previously described, using antibody panels in Table S1.[21] All cases had a blood sample submitted and 12 cases also had lymph node aspirates submitted. Expression of CD21, class II MHC, and CD25 on B-cells was determined for all cases submitted after May 11, 2012 (details in Appendix S1).

2.4 | Clonality testing

The PARR assay was performed as previously described.[22] A small proportion of cases (13%) had PARR requested at the time of flow cytometry. For remaining cases, PARR was performed retrospectively for all cases with sample material available.

2.5 | Statistical analysis

Clinical and clinicopathologic data were summarized. To evaluate breed-specific differences, we grouped cases into 3 broad breed groups: (a) small dog breeds with a documented increased risk of BCLL,[1] (b) Boxer dogs, and (c) other breeds. Continuous variables were compared using Kruskal-Wallis or Mann-Whitney tests and categorical variables were compared using Chi-squared tests. Overall survival was calculated as days from the time of the flow cytometry diagnosis, because this date was available for all cases. Cases lost to follow-up or alive at the time of data collection were censored at the last contact date. Deaths were categorized as: (a) "BCLL related" or "possibly BCLL related" depending on the degree of suspicion, as indicated by the clinician, or if there was disease progression noted at the time of death or euthanasia; (b) "unrelated" when indicated by the clinician; or (c) "possibly unrelated" when the cause of death or euthanasia was not indicated by the clinician, BCLL disease was stable or in remission, and co-morbidity conditions existed. Treatments were categorized as: (a) no treatment, (b) corticosteroids only, (c) corticosteroids and chlorambucil, or (d) maximum tolerated dose (MTD) injectable or PO chemotherapy (including single-agent and multi-agent protocols). Treatments were described based on the initial treatment plan, the total number of chemotherapy protocols used, and the most intensive protocol used throughout BCLL treatment. When possible, best response to treatment was retrospectively evaluated based on lymphocyte cell count, status of cytopenia(s) on CBC, and status of clinical signs noted in the medical record. Responses were classified as follows: complete response (CR), no morphologically atypical cells, normal total lymphocyte count, and resolution of clinical signs; partial response (PR), >50% reduction in lymphocyte count, persistent clinical signs or both; stable disease (SDZ), <50% reduction in lymphocyte count/≤25% increase in lymphocyte count, with or without persistent clinical signs; and progressive disease (PD), >25% increase in circulating lymphocyte count, with or without worsening clinical signs.

For survival analysis, continuous risk factors were divided into groups above and below the median, except for lymphocyte count, which was grouped above and below 60 000 lymphocytes/μL, because this cutoff was clinically relevant. Individuals with missing or unknown data were excluded from analysis. Blood smear lymphocyte morphology obtained from pathology report descriptions was categorized as: (a) intermediate-to-large-sized lymphocytes or (b) small-, small-to-intermediate-, or intermediate-sized lymphocytes. Associations between patient data and survival were assessed using Kaplan-Meier log-rank tests. For Ki67 analysis and survival analysis, the "other breeds" group was further divided into small breeds (body weight < 15 kg) and medium-large breeds (>15 kg), based on the American Kennel Club breed weight chart (AKC Material, URL: akc.org/expert-advice/nutrition/breed-weight-chart/). Statistical analysis was performed using GraphPad Prism version 7 or R version 3.5.2 and 2-sided *P*-values <.05 were considered significant.

3 | RESULTS

3.1 | Clinical presentation

Medical records were obtained for 121 BCLL cases, representing small breeds with increased BCLL risk (n = 55), Boxers (n = 33), and other breeds (n = 33). Signalment, clinicopathologic data, and physical examination findings at the time of diagnosis are summarized in Table 1. The median age of all cases was 10.4 years and 55% were male. The median lymphocyte count on CBC was 39 700 lymphocytes/μL. Forty-one percent of cases were anemic, with a median hematocrit of 31.5% (interquartile range [IQR], 26%-36%; range, 18%-39%). Of 34 anemic cases with a reticulocyte count performed, 50% had a regenerative anemia based on the laboratory's reference interval (median, 119 000 reticulocytes/μL; IQR, 102 950-246 500/μL; range, 64 000-698 000/μL). Twenty-four percent of cases were thrombocytopenic with a median count of 113 000 platelets/μL (IQR, 91 500-156 000/μL; range, 30 000-198 000/μL).

Blood smear review by a clinical pathologist was available for 107 cases. Ninety-two percent of cases were described as having small-, small-to-intermediate-, or intermediate-sized lymphocytes, and of those, 93% were described as having mature chromatin patterns.

TABLE 1 Signalment data, clinicopathologic data, physical exam findings, and treatment data for 121 BCLL patients in the outcome study

	Boxers (n = 33)		Small breed dogs (n = 55)		Other breeds (n = 33)		Non-Boxers[a] (n = 88)	
	n[b] with available data	n (%) affected[c] or median (IQR)	n[b] with available data	n (%) affected[c] or median (IQR)	n[b] with available data	n (%) affected[c] or median (IQR)	n[b] with available data	n (%) affected[c] or median (IQR)
Age (years)	33	9.3 (8.3-10.7)	55	10.9 (9.1-13.2)	33	10.6 (8.4-12.5)	88	10.6 (9.0-13.0)
Male	33	15 (45)	55	26 (47)	33	25 (76)	88	51 (58)
Lymphocyte count, $x10^3/\mu L$	33	72.7 (32.9-138.1)	55	32.0 (14.3-75.0)	33	19.4 (11.5-63.5)	88	27.0 (12.8-64.6)
Anemia	33	15 (45)	55	25 (45)	33	10 (30)	88	35 (40)
Thrombocytopenia	33	12 (36)	55	10 (18)	33	7 (21)	88	17 (19)
Hyperglobulinemia	32	6 (19)	52	12 (23)	30	4 (13)	82	16 (20)
B-cell size ratio	33	0.52 (0.48–0.56)	55	0.48 (0.44–0.51)	33	0.50 (0.45–0.57)	88	0.49 (0.45–0.54)
CD25 expression (%)	30	72 (26-96)	46	55 (21-81)	23	68 (52-91)	69	62 (25-82)
Class II MHC expression (MFI)	30	127 (100-254)	46	159 (102-220)	23	212 (82-327)	69	160 (90-260)
CD21 expression (MFI)	30	46 (31-64)	46	50 (35-70)	23	42 (32-56)	69	49 (33-68)
Peripheral lymphadenopathy	32	21 (66)	52	28 (54)	33	13 (39)	85	41 (48)
Visceral lymphadenopathy	20	11 (55)	31	12 (39)	16	8 (50)	47	20 (43)
Splenic abnormalities	30	20 (67)	41	24 (59)	25	8 (32)	66	32 (48)
Hepatic abnormalities	30	9 (30)	41	17 (41)	26	6 (23)	67	23 (34)
Clinical signs at presentation	33	14 (42)	54	20 (37)	32	11 (34)	86	31 (36)
Maximum treatment[d]	32		55		33		88	
MTD chemotherapy		15 (47)		18 (33)		10 (30)		28 (32)
Steroid/chlorambucil		7 (22)		19 (35)		10 (30)		29 (33)
Steroids only		7 (22)		7 (13)		6 (18)		13 (15)
None		3 (9)		11 (20)		7 (21)		18 (20)

Abbreviations: IQR, interquartile range; MFI, median fluorescence intensity; MHC, major histocompatibility complex; MTD, maximum tolerated dose.
[a]Non-Boxer group: combined small breed and other non-Boxer cases.
[b]n, number of cases with available data.
[c]n, number of cases affected; %, percentage of cases affected among those cases with available data.
[d]The most intensive protocol pursued throughout BCLL treatment.

Nine cases had intermediate-to-large-sized lymphocytes cytologically, but these cases were included because the cells were small-sized based on flow cytometry. Other groups have highlighted differences in cell size between microscopy and flow cytometry and suggested flow cytometry is more objective for determining size.[23-25] A few cases had descriptions of atypical chromatin patterns, including smooth, fine, or stippled. Moderate numbers of cases were described as having rare cells with a faint nucleolus, but 2 were described as having consistent nucleoli. Bone marrow cytology reports were available for 11 cases, and 3 were diagnosed with lymphocytic leukemia, 2 with lymphocytosis suspicious for CLL (40%-45% small lymphocytes), 3 with mild lymphocytosis (7%-16% small lymphocytes), 1 with few atypical lymphocytes consistent with possible lymphoid neoplasia, and 2 with normal lymphocyte number and morphology.

At diagnosis, 19% (n = 22) of cases were hyperglobulinemic. Serum protein electrophoresis was performed in 8 cases. Six had a monoclonal gammopathy and 2 had a biclonal gammopathy. Additional cases developed hyperglobulinemia after diagnosis and 25% had hyperglobulinemia during the course of disease.

Peripheral lymphadenopathy was detected in 53% of cases at diagnosis and 11 developed lymphadenopathy, resulting in 62% having peripheral lymphadenopathy. Lymph node cytology was evaluated in 37 cases and 43% were diagnosed as lymphoma (small-intermediate, n = 8; intermediate-large, n = 8), 22% as possible or probable lymphoma (small-intermediate), 24% as lymphoid hyperplasia or reactive, and 11% as within normal limits. By histology (n = 3), 1 case was diagnosed with low-grade small cell lymphoma, 1 with high-grade small cell lymphoma, and 1 with probable small cell lymphoma. Fifty-four percent of cases had splenic abnormalities at diagnosis, an additional 5 cases developed splenic abnormalities (splenic abnormalities in 58% of cases), and 2 had a history of previous splenectomy. Splenic cytology was evaluated in 17 cases and 29% were diagnosed as lymphoma (small-intermediate, n = 4; intermediate-large, n = 1), 41% as possible lymphoma, and 29% as lymphoid hyperplasia. By splenic histology (n = 3), 2 were diagnosed with low-to-intermediate-grade lymphoma of small-to-medium-sized lymphocytes and 1 was diagnosed with lymphoid hyperplasia.

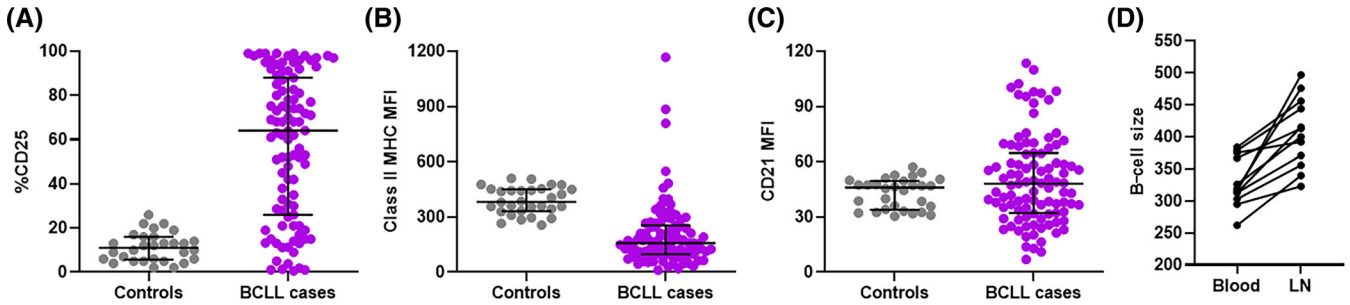

FIGURE 1 B-cell CD25, class II MHC, and CD21 expression in BCLL cases compared to control B-cells and comparison of B-cell size in paired blood and lymph node samples from BCLL cases. A–C, Expression of CD25 (A), class II MHC (B) and CD21 (C) by flow cytometry is plotted for individual cases. Lines depict the median and interquartile range for each group. BCLL cases had significantly higher CD25 expression and significantly lower class II MHC expression than B-cells from healthy controls. (D) Median forward light scatter of B-cells is plotted for paired blood and lymph node samples from BCLL cases. B-cells in the lymph node were significantly larger than those in the blood

Forty-six percent of cases had thoracic or abdominal lymphadenopathy and 33% of cases had hepatic abnormalities. Ten cases (8%) were presumed to have rectal involvement and half of these (4% of all cases) had rectal prolapse. Lymphoma was diagnosed in rectal tissue in 3 cases by cytology (n = 1) or histology (n = 2). Two (1.6%) cases had multiple dermal or SC nodules diagnosed as lymphoma by cytology. Six (5%) cases had ocular changes presumed secondary to lymphoproliferative disease or hyperglobulinemia and hyperviscosity syndrome by the clinician. Five (4%) cases had neurologic signs suspected to be secondary to lymphoproliferative disease, but only 1 case had advanced imaging performed and that dog had multifocal disease throughout the spinal cord presumed secondary to progression of lymphoproliferative disease. Seven (6%) cases had pulmonary infiltrates on thoracic radiographs interpreted as possible neoplastic infiltrate by the radiologist.

Sixty-two percent of cases were asymptomatic at diagnosis and leukocytosis with or without lymphadenopathy was an incidental finding. Among the 38% of cases with clinical signs at presentation, lethargy (n = 21) and decreased appetite (n = 20) were most common, with weight loss (n = 12), vomiting (n = 9), diarrhea (n = 9), and labored breathing (n = 6) affecting smaller numbers.

3.2 | Flow cytometry

By flow cytometry, CD21+ B-cells accounted for 64% to 99% of lymphocytes in the blood in BCLL cases. The B-cell:neutrophil size ratio ranged from 0.37 to 0.60 (median, 0.50) in BCLL cases (n = 121), compared to 0.39 to 0.54 (median, 0.44) in healthy controls (n = 30). The percentage of B-cells expressing CD25 was significantly higher in BCLL cases (median, 64%; range, 0.6%-99%; n = 99) compared to controls (median, 11%; range, 2.0%-24%; $P < .0001$; Figure 1A). B-cell class II MHC expression was significantly lower in BCLL cases (median, 158; range, 12-1168; n = 99) compared to controls (median, 383; range, 256-511; $P < .0001$; Figure 1B). B-cell CD21 expression was not significantly different between BCLL cases (median, 48; range, 7-114; n = 99) and controls (median, 46; range, 30-57; $P = .212$; Figure 1C).

Flow cytometry was performed on lymph node aspirates in 12 BCLL cases. All lymph node samples had marked expansion of small-sized B-cells, consistent with a small B-cell neoplasm. The CD21+ B-cells accounted for 88% to 97% of all leukocytes and the median forward light scatter of B-cells was <500 (median, 407; range, 323-497) in all cases. B-cells in the lymph node were larger than the same case's B-cells in the blood by flow cytometry (Figure 1D) but no consistent phenotypic differences between blood and lymph node were noted. Nine of 12 cases had flow cytometry and cytology performed concurrently on a lymph node aspirate. Cytologically, 5 cases were diagnosed as lymphoma of small-to-intermediate-sized lymphocytes (n = 1), intermediate lymphocytes (n = 1), or intermediate-to-large-sized lymphocytes (n = 3). One case was diagnosed as probable lymphoma, 2 as lymphoid hyperplasia, and 1 as normal.

3.3 | Breed differences

Clinical and clinicopathologic data were compared among 3 breed groups: small breeds with increased BCLL risk, Boxer dogs, and other breeds without increased risk of BCLL. No significant differences were found between small breeds with increased risk and the "other breeds" group, except for sex; therefore, small breed and "other breed" cases were combined into a non-Boxer group (n = 88). Comparing Boxer and non-Boxer cases, Boxers were significantly younger (median, 9.3 years) than non-Boxers (median, 10.6 years; $P = .01$) and Boxers had a higher presenting lymphocyte count (median, 72 700/μL) than did non-Boxers (27 000/μL; $P < .001$; Figure 2A). No difference was observed in the frequency of anemia, thrombocytopenia, hyperglobulinemia, lymphadenopathy, or splenic changes. By flow cytometry, Boxers had significantly larger B-cells compared to non-Boxers ($P = .04$; Figure 2B). B-cell CD21 and class II MHC expression was not significantly different. The median percentage of CD25-expressing B-cells was not significantly different, but a larger proportion of Boxers (20%) had very high CD25 expression (≥98% CD25-expressing B-cells) compared to non-Boxers (4%; $P = .04$; Figure 2C).

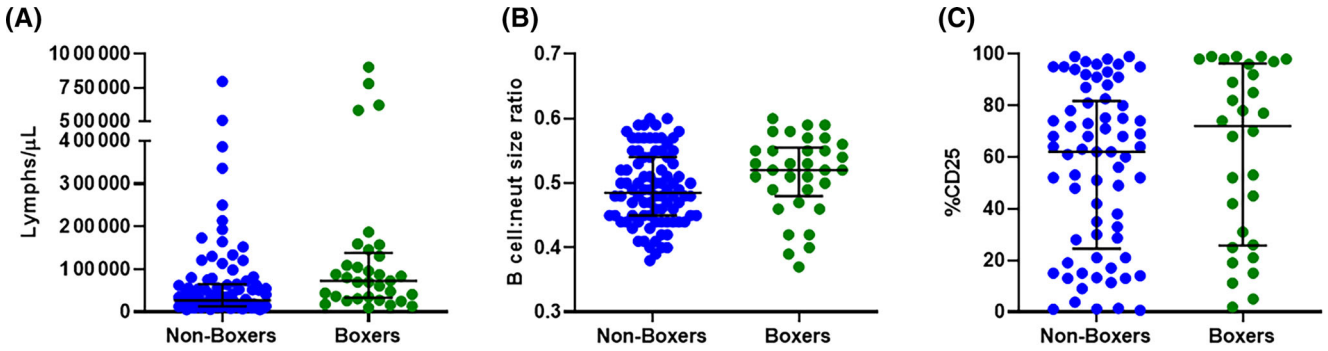

FIGURE 2 Breed-specific differences in lymphocyte count, B-cell size, and B-cell CD25 expression between Boxers and non-Boxers with BCLL. Presenting lymphocyte count (A), B-cell:neutrophil forward side scatter ratio (B), and B-cell CD25 expression (C) are plotted for non-Boxer and Boxer BCLL cases. Lines depict the median and interquartile range for each group. Boxers had significantly higher presenting lymphocyte counts and larger-sized B-cells than non-Boxers. There was a significantly larger proportion of Boxer cases with very high CD25 expression (≥98% CD25-expressing B-cells) compared to non-Boxers

3.4 | Clonality

Eighty-seven cases had PARR analysis, including on blood in 84 of 87 cases, splenic aspirate in 2 cases, and lymph node aspirate in 1 case. Eighty-five (98%) of 87 cases had clonal immunoglobulin rearrangements, supporting the diagnosis of B-cell neoplasia.

3.5 | Ki67 expression

Independent of the outcome study, the percentage of CD21+ B-cells expressing Ki67 (Ki67%) was determined by flow cytometry in controls (n = 12), BCLL cases (n = 202), and nodal B-cell lymphoma cases (B-cell size >500; n = 101) collected from 2017 to 2019. The BCLL cases included small breeds with increased risk of BCLL[1] (n = 84), other small breeds (n = 32), non-Boxer medium-large breeds (n = 38), and Boxers (n = 48). A subset of those BCLL cases (n = 39) then was included in the outcome study. Low Ki67% expression was defined as <20% Ki67-expressing B-cells and high Ki67% as >40% Ki67-expressing B-cells.[12] No significant difference in Ki67 expression was found between normal B-cells in the control blood (n = 5) and lymph node (n = 7) samples, and consequently these samples were combined for analysis. The median Ki67% in control B-cells was 8.5% and 11 of 12 control cases had low Ki67 (<20%) expression (Figure 3). Among non-Boxer BCLL breed groups, median Ki67% ranged from 14% to 16% and no significant differences were found among small breeds with increased BCLL risk, other small breeds, and non-Boxer medium-large breeds. Boxers with BCLL had significantly higher Ki67% (median, 42%) compared to non-Boxer BCLL cases ($P < .0001$). Nodal B-cell lymphoma cases (B-cell size >500) had significantly higher Ki67% (median, 64%) than all BCLL groups ($P < .0001$), and 87% of these nodal B-cell lymphoma cases had high Ki67 (>40%) expression. The percentage of high Ki67 (>40%) cases was significantly higher in Boxers with BCLL (58%) compared to non-Boxers with BCLL (27%; $P < .0001$), and the majority of non-Boxer BCLL cases (59%) had low Ki67 (<20%).

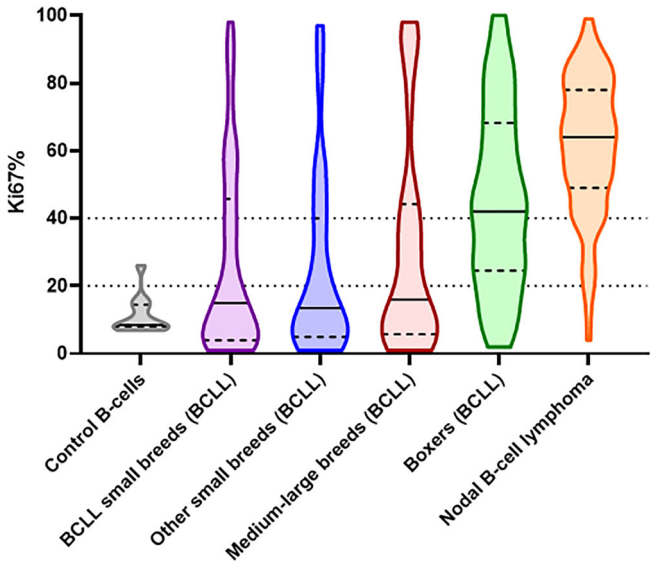

FIGURE 3 B-cell Ki67% expression measured by flow cytometry in control B-cells, BCLL cases, and nodal B-cell lymphoma cases (B-cell size >500). The percentage of CD21+ B-cells expressing Ki67 is on the vertical axis. Low Ki67 expression was defined as <20% Ki67-expressing B-cells and high Ki67 expression as >40% Ki67-expressing B-cells. The majority of non-Boxer BCLL cases had <20% Ki67 and there was no significant difference in Ki67 expression between small breeds with increased BCLL risk, other small breeds, and non-Boxer medium-large breeds. Boxers with BCLL had significantly higher Ki67%. The vast majority of nodal B-cell lymphoma cases (B-cell size >500) had high Ki67 (>40%). Violin plots show the median (solid line) and quartiles (dashed lines) for each group

3.6 | Clinical outcome and survival analysis

The median survival time (MST) from flow cytometry diagnosis for all 121 outcome cases was 300 days (range, 1-1644 days). Boxers had significantly shorter survival (MST, 178 days) compared to non-Boxers (MST, 423 days; $P < .0001$; Figure 4A). No significant difference in

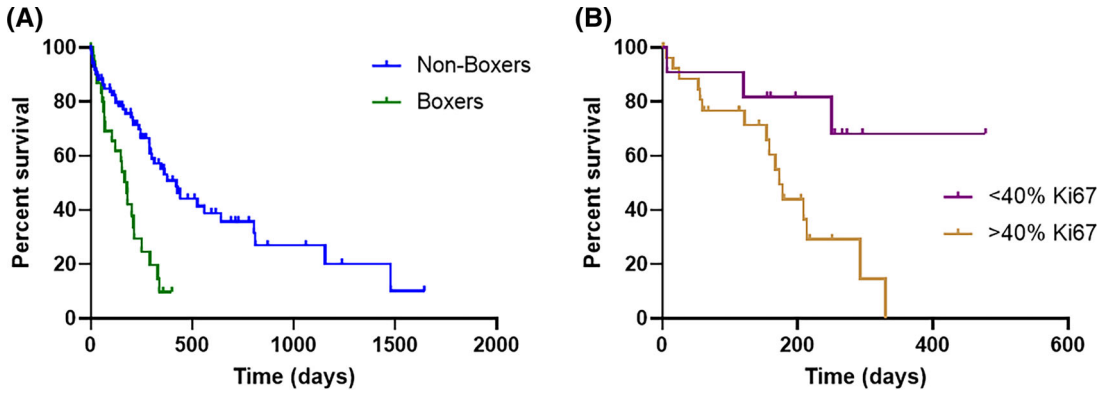

FIGURE 4 Kaplan–Meier curves showing overall survival in cases with BCLL. A, Boxers had significantly shorter survival than non-Boxers. B, Cases with high Ki67 (>40%) had significantly shorter survival than cases with low-intermediate Ki67 (<40%)

survival was found between small breed dogs and non-Boxer medium-large breed dogs ($P = .46$), and consequently all non-Boxer cases were combined for further analysis. Treatment across cases varied considerably (Table 1). Chemotherapeutics included chlorambucil, cyclophosphamide, vincristine, doxorubicin, mitoxantrone, L-asparaginase, mustargen, vinblastine, rabacfosadine, lomustine, melphalan, cytarabine, procarbazine, and dacarbazine. Across all dogs, first treatment pursued included no treatment for 30 dogs, corticosteroid only for 24 dogs, corticosteroid and chlorambucil for 41 dogs, and MTD chemotherapy for 24 dogs. For those dogs receiving chemotherapy at some point in their disease course, 48 dogs received a single chemotherapy protocol, 20 dogs received 2 chemotherapy protocols, and 11 received ≥3 chemotherapy protocols. For those dogs treated with corticosteroid alone as their first treatment, 9 of 24 had information available to retrospectively determine best response, and 1 dog had a CR, 4 dogs had PR, and 4 dogs had SDZ. For those dogs treated with corticosteroid and chlorambucil as their first treatment, 36 of 41 had a best response determined, and 39% had a CR, 39% had PR, and 22% had either SDZ or PD. For those dogs treated with MTD chemotherapy as their first treatment, 22 of 24 had a best response determined, and 41% had a CR, 55% had PR, and 1 dog had SDZ. It was not always clear why MTD chemotherapy was chosen for first treatment, but factors included marked lymphocytosis, recent rapid progression of lymphocytosis, and evidence of lymphoproliferative disease in solid tissues suggesting disseminated disease. Cases with peripheral lymphadenopathy ($P = .001$) or splenic abnormalities ($P = .002$) received MTD chemotherapy more frequently than did cases without abnormalities in those sites.

Among 67 cases that died or were euthanized, 30 (45%) cases had a BCLL-related death, 12 (18%) had a possible BCLL-related death, 3 (4%) had an unrelated death, 5 (7%) had a possible unrelated death, and in 17 (25%) cases the cause of death was unknown.

The Ki67 data were available for 39 cases with survival data, including 15 Boxers and 24 non-Boxers. High Ki67% (>40%) cases had significantly shorter survival (MST, 173 days; n = 28) compared with cases with <40% Ki67 (MST, undefined; n = 11; $P = .03$; Figure 4B). The 3 cases with <40% Ki67 expression that died included a non-Boxer with heartworm disease and bicavitary effusion that died at home and the only 2 Boxers in the <40% Ki67 group, which were both managed aggressively using multi-agent MTD protocols. When Boxers were omitted from Ki67 survival analysis, the results did not change, indicating that non-Boxers with high Ki67% also have poorer prognosis. Low-intermediate Ki67 cases (<40% Ki67) were managed with corticosteroids, chlorambucil or both (36%), or no treatment (36%), except for the 2 Boxers in the group (18%, MTD chemotherapy) and 1 non-Boxer that received lomustine for a lung tumor. Among high Ki67 cases (>40% Ki67), 46% received MTD chemotherapy, 43% received corticosteroids with or without chlorambucil, and 11% were untreated. Of those dogs with <40% Ki67 treated with chemotherapy (n = 6), only 2 dogs were treated with >1 protocol. Of those dogs with >40% Ki67 treated with chemotherapy (n = 16), 8 dogs were treated with >1 protocol.

Clinical and laboratory variables were assessed for association with survival in all cases, non-Boxer cases only, and Boxer cases only (Table 2). Among all cases, high presenting lymphocyte count, presence of clinical signs at diagnosis, and high CD25 expression were associated with significantly shorter survival. Presence of hyperglobulinemia was associated with significantly longer survival. Among non-Boxers only, lymphocyte count, clinical signs, and hyperglobulinemia were still similarly associated with survival, but CD25 expression was no longer associated with survival. Among Boxers only, presence of clinical signs and absence of splenomegaly or splenic masses were associated with significantly shorter survival. Across all cases, although the number was small, cases with lymphocytes described as intermediate-large in size by microscopy (n = 9) had significantly shorter survival than cases described as having lymphocytes that were small, small-intermediate, or intermediate in size. When cases with intermediate-sized lymphocytes were combined in the intermediate-large group, this finding was no longer significant. Six of the 9 cases with intermediate-large-sized lymphocytes were Boxers.

TABLE 2 Log-rank test results evaluating factors for potential association with survival

		All cases (n = 121)			Non-Boxers (n = 88)			Boxers (n = 33)		
Variable	Cutoff	Number of cases	MST (days)	*P*	Number of cases	MST (days)	*P*	Number of cases	MST (days)	*P*
Age	≥10 years	70	364	.46	57	423	.38	13	181	.25
	<10 years	51	238		31	804		20	167	
Sex	Male	66	330	.11	51	441	.55	15	181	.19
	Female	55	250		37	377		18	154	
Lymphocyte count	≥60 000	45	173	<.001 *	25	173	<.001*	20	178	.70
	<60 000	76	423		63	526		13	167	
Anemia	Present	50	288	.94	35	314	.48	15	250	.14
	Absent	71	300		53	423		18	149	
Thrombocytopenia	Present	29	210	.21	17	238	.59	12	210	.98
	Absent	92	337		71	423		21	154	
B-cell size (flow cytometry)	≥0.50	63	289	.42	40	423	.91	23	178	.66
	<0.50	58	364		48	377		10	95	
Lymphocyte size (blood smear)	Intermediate-large	9	154	.002*	3	59	.10	6	160	.53
	Small-intermediate	98	346		75	427		23	181	
Hyperglobulinemia	Present	22	804	.02*	16	Undefined	.03*	6	250	.12
	Absent	92	289		66	364		26	178	
Peripheral lymphadenopathy	Present	62	245	.09	41	300	.09	21	178	.92
	Absent	55	423		44	642		11	167	
Visceral lymphadenopathy	Present	31	250	.15	20	292	.09	11	250	.78
	Absent	36	337		27	526		9	202	
Splenic abnormalities	Present	52	300	.57	32	423	.58	20	214	.02*
	Absent	44	288		34	364		10	149	
Clinical signs	Present	45	149	.003*	31	200	.03*	14	68	.005*
	Absent	74	346		55	427		19	210	
CD25 expression	≥64%	50	202	.02*	33	314	.19	17	104	.05
	<64%	49	377		36	427		13	272	
CD21 expression (MFI)	≥48	50	364	.12	36	526	.04*	14	167	.44
	<48	49	207		33	300		16	178	
Class II MHC expression (MFI)	≥158	50	250	.57	37	364	.58	13	167	.51
	<158	49	314		32	427		17	178	
Initial treatment[a]	MTD chemo	24	214	.01*	14	314	.22	10	202	.01*
	Steroid/chlorambucil	41	441		31	559		10	330	
	Steroids only	24	125		15	245		9	68	
	None	30	423		27	427		3	210	
Maximum treatment[b]	MTD chemo	43	250	.11	28	314	.3	15	202	.02*
	Steroid/chlorambucil	36	526		29	526		7	167	
	Steroids only	20	178		13	364		7	69	
	None	21	293		18	423		3	210	

Note: Undefined: median survival time not reached; **P*-value <.05 was considered significant.

Abbreviations: MFI, median fluorescence intensity; MHC, major histocompatibility complex; MST, median survival time (days); MTD, maximum tolerated dose chemotherapy; n, number of cases.

[a]Treatment category represents the first protocol used.

[b]Treatment category represents the most intensive protocol pursued.

4 | DISCUSSION

Our study found that BCLL, defined by an expansion of small-sized CD21+ B-cells in the blood by flow cytometry, had variable clinical presentation and outcome. Cases had wide variability in overall survival times, and Ki67 expression was useful in identifying cases with a poorer prognosis. Additionally, Boxers with BCLL had significantly shorter overall survival.

The overall survival time for all 121 BCLL cases was 300 days (range, 1-1644 days); however, Boxers were intentionally overrepresented in this population. When survival was assessed in non-Boxers, the MST of 423 days was similar to that previously reported for dogs with BCLL.[3] A subset of cases had more indolent disease and were frequently asymptomatic. These cases were managed with corticosteroids, corticosteroids and chlorambucil, or no treatment, and had prolonged survival. Among non-Boxers, cases treated with corticosteroids and chlorambucil often had prolonged survival (MST, 526 days; range, 2-1478 days). However, a subset of BCLL cases had more aggressive disease and shorter overall survival. We aimed to find factors that would help identify these cases.

B-cell Ki67 expression, using cutoffs from a previous study in dogs,[12] was variable in BCLL cases and associated with prognosis. Most non-Boxers had low-intermediate Ki67 expression (Ki67% <40%), but a subset of non-Boxers and most Boxers had high Ki67 expression (Ki67% >40%). Cases with high Ki67 expression, indicating increased cellular proliferation, had significantly shorter survival. Most nodal B-cell lymphoma cases (B-cell size >500) had Ki67% >40%. These nodal cases did not have histology, but most cases (87%) with B-cell forward scatter >500 are diffuse large B-cell lymphoma.[20] We found that peripheral blood B-cells in healthy control dogs and the majority of non-Boxer BCLL cases had low Ki67% (<20%). We did not have enough low-intermediate Ki67 BCLL cases with outcome to determine whether low Ki67 cases (Ki67% <20%) and intermediate Ki67 cases (Ki67% 20%-40%) have different prognoses, but rather, these cases were combined for analysis.

We hypothesized that Boxers with BCLL have more aggressive disease, because of preferential use of unmutated immunoglobulin heavy variable region genes in neoplastic B-cells,[16] which is associated with poor prognosis in humans with CLL.[17,18] Other factors, either in combination or regardless of immunoglobulin gene mutation status, could contribute to more aggressive disease in Boxers. In humans with CLL, there is a wide spectrum of identified mutations, and certain mutations, including SF3B1 and TP53 mutations, are associated with poorer prognosis.[26] Boxers may have mutations associated with aggressive disease more frequently than other breeds. In our study, Boxers had significantly higher presenting lymphocyte counts, higher Ki67 expression, and shorter overall survival compared to non-Boxers. Additionally, we examined whether other medium-large breed dogs had poorer prognosis similar to Boxers, but these other breeds had similar Ki67 expression and survival as small breed dogs.

We hypothesize that BCLL in dogs can have different manifestations, as seen with CLL in humans, and Ki67 expression is useful in distinguishing subsets with different outcomes. These different subsets may have different small cell B-cell neoplasms, rather than different clinical manifestations of BCLL. Alternatively, all cases may have BCLL and the disease has a high degree of molecular, genetic, and clinical heterogeneity as seen in CLL in humans.[27] A subset of human CLL patients has "accelerated CLL," characterized by expanded proliferation centers in lymph nodes with a high proliferation rate and associated with aggressive disease.[13] Gene expression profiling and identification of driver mutations would be useful in determining whether these canine BCLL cases represent a spectrum of a single disease or distinct diseases. Currently, antibodies are not available to differentiate these BCLL subsets by routine immunophenotyping, because our canine B-cell antibodies are limited[28] and class II MHC and CD21 expression was variable across subsets and substantial overlap in CD25 expression was found among groups.

In addition to Ki67 expression and breed, we found that lymphocyte count, presence of clinical signs, and hyperglobulinemia were associated with prognosis. A high presenting lymphocyte count (>60 000/μL) was associated with shorter survival in non-Boxer BCLL cases. Lymphocyte count was not prognostic in 2 prior studies of BCLL in dogs.[3,11] These studies may not have detected a difference because of smaller sample sizes ($n = 17$ and $n = 21$), or because they assessed association with survival using a different lymphocyte cutoff (30 000/μL) or lymphocyte count as a continuous variable. In our study, lymphocyte count was not prognostic when using the median lymphocyte count of the study population as a cutoff (40 000/μL), but was prognostic when the cutoff was increased to 60 000/μL. Those BCLL cases presenting with clinical signs had significantly shorter survival, which is an important prognostic factor in other types of lymphoma in dogs.[29,30] Hyperglobulinemia affected 19% of canine BCLL cases at diagnosis and was associated with longer survival. In humans with CLL, paraproteins are associated with poor prognosis.[31-33] It was surprising that canine BCLL cases with hyperglobulinemia had prolonged survival, but only 8 cases had serum protein electrophoresis and it is unknown whether the hyperglobulinemia was attributed to a paraprotein in all cases. If paraprotein cases have prolonged survival, perhaps those cases have different signals driving B-cell stimulation or the neoplastic B-cells are more differentiated, affecting the clinical disease course. High CD25 expression was associated with shorter survival across all cases, but not when assessed in non-Boxers only. Increased CD25 expression is associated with poor prognosis in dogs with diffuse large B-cell lymphoma, but more work is needed to fully elucidate its prognostic relevance in dogs with BCLL.[20] Age was not prognostic in our study as it was in a prior study.[3] Most cases in our study were middle-aged to older (only 7/121 [6%] were <6 years old), and perhaps too few young cases were available to detect a difference in survival.

We included all cases with a flow cytometry diagnosis of BCLL, regardless of tissue involvement. In humans, CLL commonly affects secondary lymphoid tissues and SLL, which is defined by lymphadenopathy, splenomegaly, or both, is considered the same neoplasm as CLL without a leukemic component.[34] Therefore, we did not want to exclude canine BCLL cases with secondary lymphoid organ enlargement and found that lymphadenopathy and splenic changes were common. These cases more often received MTD chemotherapy.

All 12 cases with flow cytometry performed on a lymph node aspirate had a marked expansion of small-sized B-cells, consistent with neoplasia. These cases had a wide range of cytology diagnoses, emphasizing the difficulty in diagnosing small cell neoplasms by cytology alone. In the peripheral blood, lymphocyte cell size also was variable across pathology reports. It was not unusual for cases to be described as having intermediate-sized lymphocytes; these cases had small B-cell size by flow cytometry and had the same prognosis as cases described as small-sized. These findings suggest that BCLL tumors may have intermediate-sized lymphocytes described using lymph node or blood microscopy and this morphology does not necessarily indicate a different tumor or prognosis. Nine cases had intermediate to large-sized lymphocytes in the blood by microscopy and these cases did have a poorer prognosis. These cases may represent more aggressive BCLL disease or a different B-cell neoplasm. Nuclear descriptions were variable or lacking in these cases, making it difficult to fully characterize the cells. Future studies that objectively characterize morphology on fresh blood films are needed to evaluate the prognostic utility of morphologic features. In humans, CLL may have admixed larger cells and up to 55% prolymphocytes, and cases with increased prolymphocytes have poorer prognosis.[35] A previous study of CLL in dogs found that prolymphocytes were not prognostic, but only 1 of 17 BCLL cases had >10% prolymphocytes.[3]

Limitations of our study included its retrospective study design, incomplete staging for some cases, variable treatment protocols, and lack of necropsies. Additionally, by including cases with tissue involvement, atypical cellular morphology or both, we may have included cases with a different small cell B-cell neoplasm. For example, 27% of cases with a histologic diagnosis of diffuse small B-cell lymphoma had lymphocytosis, suggesting some cases may present similarly to BCLL.[36] However, we currently cannot differentiate subtypes of small cell B-cell neoplasms by immunophenotyping, and therefore wanted to include any cases with a diagnosis of BCLL by routine flow cytometry. Additionally, not all cases with lymphadenopathy had cytology, and few had nodal histology or flow cytometry. Therefore, we cannot confirm whether cases developed diffuse large B-cell lymphoma or another aggressive lymphoma (Richter syndrome). Richter syndrome is reported in 2% to 10% of human CLL cases and 11% of canine BCLL cases.[2,37] Thus, in the more aggressive cases in our study, we could not determine whether cases had Richter transformation or progressive BCLL. In conclusion, BCLL in dogs, as defined by flow cytometry, has a variable clinical course. Future work to evaluate differences in gene expression and mutations among subsets with different clinical behavior will be particularly important.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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