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Evaluation of serum thymidine kinase 1 activity as a biomarker for treatment effectiveness and prediction of relapse in dogs with non-Hodgkin lymphoma

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Abstract

Background: Serum thymidine kinase 1 (sTK1) activity is closely correlated with DNA synthesis.

Objectives: Evaluate sTK1 activity as a biomarker for treatment response and early detection of relapse in dogs with lymphoma.

Animals: Ninety-seven client-owned dogs with naive or relapsed lymphoma and 23 healthy dogs.

Methods: Prospective study. Serum TK1 activity measured by refined ELISA-based method (DiviTum assay, Biovica International) before treatment, at clinical response, and every 4 weeks until relapse or last follow-up.

Results: Serum TK1 activity was ≤20 Du/L in 96% (22/23) of healthy dogs. Pretreatment sTK1 activity was >20 Du/L in 88% (85/97) dogs with lymphoma. At clinical response, sTK1 activity was significantly lower in dogs with complete (CR, n = 36) versus partial (PR, n = 29) response (P < .0001). Sensitivity (Se) and specificity (Sp) of sTK1 activity for detecting nonfully responders were 76% and 100%, respectively, with cutoff of 119.5 Du/L (AUC, 0.90; 95%-CI, 0.81-0.98; P < .0001). In dogs with CR, a 5-fold increase in sTK1 activity at a 4-week interval predicted relapse at the subsequent 4-week assessment with a Se 50% and Sp 94% (AUC, 0.72; 95%-CI, 0.55-0.90; P = .02). An increase of sTK1 activity (>2.7-fold value measured at clinical response) predicted relapse at subsequent 4-week assessment with a Se 61% and Sp 88% (AUC, 0.79; 95%-CI, 0.64-0.95; P = .004).

Conclusions and Clinical Importance: Monitoring sTK1 activity could help to detect complete responders and early disease progression in dogs with lymphoma.

KEYWORDS
chemotherapy, DiviTum assay, monitoring, remission

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1 | INTRODUCTION

Non-Hodgkin lymphomas (NHL) are among the most common hematopoietic cancers reported in dogs.[1-4] Most studies show that NHL represent approximately 12% to 18% of all canine malignancies with an annual incidence up to 107 per 100 000 dogs.[1,2,5-10] Multiagent chemotherapy (anthracycline-based protocol) is considered the standard of care for treatment of NHL with an overall response rate between 65% and 98%.[11-23] Despite an initial response, nearly 80% to 90% of dogs do not achieve durable remission and develop relapsed/refractory disease. Evaluating remission status and detecting subclinical progressive disease in treated dogs after initial response remain challenging. Many clinical and biological factors have prognostic value in duration of remission and survival in lymphoma in dogs, including WHO clinical stage[24,25] and substage,[11,24,26] immunophenotype,[14,24,27-30] histopathological grade,[24,29,31] cytomorphological subtype,[32,33] anatomical location[34-36] and prior steroid treatment.[29,37] Other potential prognostic markers have been evaluated such as argyrophilic nucleolar organizing regions (AgNOR staining),[38] Ki-67 antibody staining,[39] and serum lactate dehydrogenase (LDH) activity.[40,41] However, these prognostic indicators do not accurately evaluate remission status; a blood marker able to evaluate treatment effectiveness and predict relapse before clinically detectable disease would be an attractive tool.

Many hematopoietic malignancies are characterized by a very high proliferation rate. Thymidine kinase is a cytoplasmic enzyme that catalyzes the phosphorylation of thymidine to thymidine monophosphate and its expression is closely correlated with DNA synthesis and cell proliferation.[42-45] Two forms of thymidine kinase have been described: cytoplasmic thymidine kinase 1 (TK1) and mitochondrial thymidine kinase 2 (TK2). Thymidine kinase 1 is associated with cellular proliferation, whereas TK2 is needed for mitochondrial DNA precursor synthesis. Thymidine kinase 1 activity increases markedly after the G1-S transition in the cell cycle and then declines rapidly in G2.[42,46,47] Any increase in extracellular TK1 activity could thus reflect an overall increase in DNA synthesis and the number of cells dying in the replicative stage of the cell cycle, releasing TK1 into the blood.[48,49]

In humans, sTK1 activity provides information regarding prognosis and treatment effectiveness in hematologic malignancies.[50-52] In dogs, sTK1 activity is significantly higher in dogs with lymphoma compared to healthy dogs and dogs with inflammatory diseases or nonhematologic tumors.[49,53] Mirroring human patients, sTK1 activity decreases significantly when dogs experience an objective response after treatment initiation and increases again at the time of relapse.[49,53-55]

We hypothesized that sTK1 activity would be lower in lymphoma-bearing dogs that fully responded to treatment and would increase after initial complete response (CR) in dogs with progressive disease before clinically detectable relapse. The primary objective of this study was to prospectively evaluate sTK1 activity as a biomarker for treatment response and early detection of relapse in dogs with naive or relapsed, intermediate to high-grade, NHL treated with nonstandardized chemotherapy. The secondary objectives were to investigate the correlation between pretreatment sTK1 activity with WHO clinical stage and substage, immunophenotype, cytomorphological subtype of the disease, and pretreatment plasma LDH activity.

2 | MATERIALS AND METHODS

2.1 | Dogs selection

Between August 2015 and March 2017, serum samples were prospectively collected from 97 client-owned dogs presented to Oncovet, Villeneuve d'Ascq, France, with a histologically or cytologically confirmed diagnosis of NHL. Dogs were considered eligible to be enrolled in the study when they (i) had a new or previously diagnosed intermediate to high-grade NHL, (ii) had measurable disease at the time of inclusion, (iii) had received no anticancer treatment in the month before the inclusion (including steroids), (iv) had no severe biochemical abnormalities or cytopenia, which precluded the use of cytotoxic drugs, and (v) had no concurrent serious systemic disorders at the time of inclusion. Information including breed, age, sex, body weight, prior treatment administered, and medical history were recorded for all dogs. In addition, sTK1 activity was measured in 23 healthy dogs as a control group. Dogs were considered healthy if they had no clinical signs of any disease at the time of the blood collection and no medical history and treatment over the last 6 months before inclusion. This study protocol was approved by the OCR Ethical Committee, and samples were collected with informed owner consent.

2.2 | Initial staging

At presentation, all dogs were staged based on the modified WHO 5-stage criteria for canine lymphoma, and lymph node size was measured using the Veterinary Cooperative Oncology Group (VCOG) Response Evaluation Criteria for Peripheral Nodal Lymphoma in Dogs v1.0.[56] Initial staging tests included CBC, biochemistry panel, ionized calcium, thoracic radiographs, abdominal ultrasound, liver and spleen cytology, urine analysis, and bone marrow aspirate. Bone marrow was considered infiltrated if neoplastic lymphoid cells represented ≥3% of all nucleated cells on the basis of bone marrow cytology.[57] Definitive diagnosis for each dog was confirmed on lymph node cytology by a board-certified clinical pathologist and graded according to the updated Kiel morphological classification.[58] Immunocytochemistry was performed on lymph node aspirates using antibodies targeting CD-3, used as a pan T-cell marker (monoclonal mouse anti-human F7.2.38; Dako, Denmark) and an antibody targeting CD-20, used as a pan B-cell marker (rabbit anti-human RB-9013-P; Thermoscientific, Waltham, Massachusetts). Neoplastic cells negative for both CD20 and CD3 were also evaluated for PAX5 (clone 24; Cell Marque, Rocklin, California), and BLA36 (clone A27-42; Biogenex, Fremont, California) expression. Cytology and immunophenotype were recorded for all dogs.

2.3 | Treatment and assessment of response

Seventy-five of 97 dogs (77%) received maximum tolerated dose chemotherapy using 1 or more of several types of protocol. Response to treatment was assessed before each chemotherapy administration based on peripheral lymph node size measurement using a caliper. A clinical evaluation was performed every 4 weeks after completion

of discontinuous chemotherapy protocol. Response to treatment was determined according to the VCOG criteria v1.0.[56] A CR was defined as the disappearance of all measurable disease (ie, lymph nodes returned to a normal size considered nonpathologic in the judgment of the evaluator and no new sites of disease should be observed). A partial response (PR) was defined as at least 30% reduction in the sum of the widest diameters of peripheral lymph nodes measured at presentation. Stable disease was defined after 21 days of treatment as <30% reduction or <20% increase in the sum of the widest diameters of the peripheral lymph nodes measured at presentation. Progressive disease was defined as >20% increase in the sum of the widest diameters of measurable peripheral lymph nodes or the appearance of new lesions. Only dogs with CR or PR were classified as experiencing an objective clinical response.

2.4 | DiviTum assay of serum TK1 activity measurement

Serum samples were collected for sTK1 activity measurement in 97 lymphoma-bearing dogs before any treatment. Of 97 dogs, 22 (22%) had only 1 sTK1 activity measured at inclusion and the 75 that received chemotherapy had sTK1 activity measured again at the time of the best clinical response. In dogs with CR, additional serum samples were collected at 4-week intervals until clinical relapse or last clinical evaluation. Serum samples were also collected for sTK1 activity measurement in 23 healthy dogs. Serum samples were centrifuged, and a minimum of 0.5 mL of serum was banked and stored at −20°C within 1 hour of sample collection. Samples were run in batches depending on the number of samples and transported in ice pack by express/overnight service. Analysis of TK1 activity was determined by a refined enzyme-linked immunosorbent assay (ELISA), the DiviTum assay (Biovica International, Uppsala, Sweden), according to the manufacturer's instructions (http://biovica.com/), as previously described.[59-64] Serum was added to reaction mixture, containing bromodeoxyuridine (BrdU), in a 96-well ELISA titer plate. Thymidine kinase 1 activity generates BrdU triphosphate which can be measured using anti-BrdU monoclonal antibody conjugated to alkaline phosphatase. The absorbance readings to DiviTum units per liter (Du/L) were converted using the values from standards with known TK1 activity, with a minimum detectable activity for this assay of 20 Du/L. The analyses were performed at the Biovica Laboratory in Uppsala, Sweden, and investigators were blinded to dog and tumor data.

2.5 | Statistical analysis

Objective clinical response to treatment and clinical relapse were the primary end points, evaluated according to the VCOG Response Evaluation Criteria for Peripheral Nodal Lymphoma in Dogs v1.0.[56] Continuous data was expressed as median and range, and categorical data as frequencies and percentages. Serum TK1 activity was compared among subsets of dogs using a Mann-Whitney rank-sum test or Kruskal-Wallis 1-way analysis of variance on ranks as appropriate. Evaluation of correlation between sTK1 activity and plasma LDH activity was performed using a Pearson correlation coefficient. A receiver operating characteristic (ROC) curve was used to determine the area under the curve (AUC) and select the optimum cutoff value that maximized the Youden's J statistic (sensitivity + specificity − 1) for sensitivity and specificity reporting. P-values ≤.05 were considered significant. All statistical analysis was performed with SAS 9.4 software (SAS Institute, Cary, North Carolina).

3 | RESULTS

3.1 | Study population

Ninety-seven client-owned dogs with naturally occurring intermediate- to high-grade NHL were included in this study. Epidemiologic and clinical characteristics are summarized in Table 1.

Of 97 dogs, 89 (92%) had a complete initial clinical staging, according to the modified WHO classification for canine lymphoma. Staging was incomplete in 8 (8%) dogs because of financial reasons or owners' wishes. Twenty-four (25%) dogs had a confirmed WHO clinical stage V disease based on bone marrow aspirates. Seventy-five dogs (77%) dogs were diagnosed with a B-cell lymphoma and 15 (15%) dogs with a T-cell lymphoma. The most common lymphoma subtypes (58/97, 60%) were diffuse large B-cell lymphoma (DLBCL), 32 as centroblastic polymorphic and 26 as immunoblastic. Seven other B-cell lymphomas cases were identified as late-stage marginal zone lymphomas in transition into DLBCL. The remaining B-cell lymphoma subtypes were 3 lymphoblastic lymphomas, 2 plasmacytoid lymphomas, 1 Burkitt-like lymphoma, and 1 transformed follicular lymphoma. T-cell lymphoma subtypes were 1 T-cell lymphoblastic lymphoma, 2 pleomorphic large-cell lymphomas, 1 large granular cell lymphoma, and 7 late-stage small to medium size clear-cell lymphomas. Lymphoma subtype could not be determined in 14 (14%) dogs, with the diagnosis of intermediate- to high-grade NHL based on the cytologic examination. Only 1 dog had hypercalcemia at the time of inclusion.

Sixty-four (66%) dogs had no prior treatment before being enrolled in the study, and 33 (34%) dogs had been treated with chemotherapy (27 with CHOP-based protocol, 6 with a clinical trial single-agent cytotoxic drug, including etoposide phosphate or F14512). After enrolment, 75 (77%) dogs received discontinuous maximum tolerated dose chemotherapy. Forty-one dogs received a 12-week free-maintenance CHOP-based protocol,[14] and 34 dogs received a single-agent free-maintenance protocol including doxorubicin (n = 3), mitoxantrone (n = 1), lomustine (n = 1), or a clinical trial cytotoxic drug (n = 29) using etoposide phosphate or F14512.[65,66]

Twenty-three healthy dogs were included as a control group (11 females, 12 males: 16 different breeds and 5 cross-breeds). The median age of the healthy control group was 4 years (range, 1-12 years), and the median weight was 25 kg (range, 4-55 kg). No significant difference was noted between the healthy and lymphoma group in terms of sex (P = .90) and weight (P = .13). Healthy dogs in the control group were significantly younger than dogs in the lymphoma group (median, 4 versus 7 years, P < .0001).

3.2 | Serum TK1 activity in healthy dogs

In the healthy control group, 22 of 23 (96%) dogs had their sTK1 activity ≤20 Du/L. Only 1 dog presented an sTK1 activity above 20 Du/L, measured at 101 Du/L. Epidemiologic characteristics and sTK1 activity levels are summarized in Table S1.

TABLE 1 Epidemiological and clinical characteristics of dogs with non-Hodgkin lymphoma

Epidemiological and clinical characteristics	Population studied
Sex	
Female	43.3% (45/97)
Male	53.6% (52/97)
Age, median (range), y	7 (2-19)
Body weight, median (range), kg	30 (8-65)
Breed	Golden Retriever (9), Labrador (9), French Bulldog (8), Boxer (7), Bernese Mountain Dog (6), German Shepherd (4), Jack Russell Terrier (4), British Spaniel (4), Bull Terrier (4), Shitzu (3), Rottweiler (2), Cocker Spaniel (2), Shar Pei (2), American Staffordshire Bull Terrier (2), Doberman (2), Dogue de Bordeaux (2), Bullmastiff Dog (2), Spanish Galgo (2), Hungarian Vizla (2)
	Yorkshire Terrier (1), Newfoundland Dog (1), Greyhound (1), Standard Poodle (1), Border Collie (1), Dogo Argentino (1), English Bulldog (1), Braque Francais (1), Hungarian Puli (1), Scottish Terrier (1), Cavalier King Charles Spaniel (1), Basset Hound Dog (1), West Highland White Terrier (1), Bloodhound Dog (1), Great Dane (1), Belgian Malinois (1)
	Cross-breed (5)
Clinical characteristics	
WHO stage	
Stage III	14.4% (14/97)
Stage IV	52.6% (51/97)
Stage V	24.7% (24/97)
WHO substage	
a	55.7% (54/97)
b	44.3% (43/97)
Immunophenotype	
B-cell	77.3% (75/97)
T-cell	15.5% (15/97)
Cytomorphological subtype	
DLBCL (centroblastic)	32/75
DLBCL (immunoblastic)	26/75
Transformed marginal zone lymphoma	7/75
Lymphoblastic	3/75
Plasmacytoid	2/75
Burkitt-like	1/75
Transformed follicular lymphoma	1/75
T-cell lymphoblastic	1/15
Pleomorphic large cell	2/15
Large granular cell lymphoma	1/15
Late-stage small to medium size clear cell lymphoma	7/15
Unclassified	14.4% (14/97)
Hypercalcemia	1% (1/97)
Prior treatment	
No treatment	66.0% (64/97)
Prior chemotherapy	34.0% (33/97)

Abbreviation: DLBCL, diffuse large B-cell lymphoma.

3.3 | Pretreatment sTK1 activity in dogs with intermediate-/high-grade NHL

Ninety-seven dogs with intermediate-/high-grade NHL had sTK1 activity measured before any treatment (Table 2). Pretreatment sTK1 activity demonstrated high variability (median, 1309 Du/L; range, 20-60 005). Eighty-five of 97 (88%) dogs presented a pretreatment sTK1 activity above 20 Du/L, and 12 of 97 (12%) dogs had a pretreatment sTK1 activity ≤20 Du/L (minimum detectable activity). Serum TK1 activity was significantly higher in dogs with

lymphoma before treatment compared to healthy dogs (median, 1309 versus 20 Du/L; P < .0001). In dogs with lymphoma, pretreatment sTK1 activity increased with more advanced clinical stage of the disease and was significantly higher in dogs with confirmed stage V lymphoma (P = .02; Figure 1). Dogs with B-cell lymphoma had higher pretreatment sTK1 activity compared with dogs with T-cell lymphoma (median, 1677 versus 46 Du/L; P < .001). Pretreatment sTK1 activities were significantly higher in serum from dogs with WHO substage b lymphoma compared to substage a (median, 2994 versus 499 Du/L; P < .001). There was no significant difference between pretreatment sTK1 activity in dogs with naive lymphoma and dogs with relapsed lymphoma who had received prior chemotherapy (P = .86). Four (4/33, 12%) dogs who had received prior chemotherapy before inclusion and 8 (8/64, 13%) dogs with naive lymphoma had a pretreatment sTK1 activity ≤20 Du/L. Dogs with DLBCL had a significantly higher pretreatment sTK1 activity compared to dogs with other cytomorphological subtypes (median, 1621 versus 223 Du/L; P = .01).

Pretreatment plasma LDH activity was measured in 52 of 97 (54%) dogs. The median plasma LDH activity was 311.5 U/L (range, 135-2411 U/L). Seventeen of 52 (33%) dogs had a pretreatment LDH activity above the reference interval (IR, 0-400 U/L). A significant correlation was observed between sTK1 activity and plasma LDH activity before treatment (Pearson correlation r = .633; 95% CI, 0.429-0.776; P < .0001; Figure 2).

3.4 | Serum TK1 activity in complete versus partial responders in dogs with pretreatment sTK1 activity >20 Du/L

Of 97 dogs with lymphoma, 75 (77%) received discontinuous maximum tolerated dose chemotherapy. Sixty-five of 75 (87%) treated dogs presented a pretreatment sTK1 activity above 20 Du/L, and the sTK1 activity was measured again at the time of their best clinical response. Based on the physical examination and VCOG criteria,[56] 36 dogs had a CR with a median time to best response of 10 days (range, 5-54 days) and 29 dogs had a PR with a median time to best response of 13 days (range, 6-69 days). Epidemiologic characteristics and sTK1 activity levels of dogs with complete and PR are summarized in Tables S2 and S3. Serum TK1 activity before treatment was significantly higher than sTK1 activity measured at the time of the best clinical response (median, 1638 versus 34 Du/L; P < .0001). Serum TK1 activity was significantly lower in dogs with CR versus PR (median, 20 versus 276 Du/L; P < .0001; Figure 3A). Serum TK1 activity levels in dogs with lymphoma that were in CR were not significantly different from the sTK1 activity levels in healthy dogs (P = .06). However, sTK1 activity in dogs with PR was significantly higher than activity measured in healthy dogs (P < .0001). The ROC curve analysis showed an AUC of 0.90 (95% CI, 0.816-0.985; P < .0001), and using a cutoff of 119.5 Du/L, the sensitivity and specificity of sTK1 activity for detecting nonfully responders were 76% and 100%,

TABLE 2 Serum thymidine kinase 1 activity measured before treatment according to clinical characteristics

Clinical characteristics	Number of dogs (%)	Pretreatment sTK1 activity, Du/L, median (range)	P-value
All population	97 (100%)	1309 (20-60 005)	
WHO stage			.02[a]
Stage III	14 (14.4%)	1236,5 (20-3972)	
Stage IV	51 (52.6%)	882 (20-60 005)	
Stage V	24 (24.7%)	3878 (20-47 453)	
WHO substage			<.001[b]
Substage a	54 (55.7%)	499 (20-16 140)	
Substage b	43 (44.3%)	2994 (20-60 005)	
Immunophenotype			<.001[b]
B-cell lymphoma	75 (77.3%)	1677 (20-60 005)	
T-cell lymphoma	15 (15.5%)	46 (20-10 476)	
Morphotype			.01[b]
DLBCL	58 (59.8%)	1621 (20-60 005)	
Others	25 (25.8%)	223 (20-5222)	
Prior treatment			.86[b]
No treatment	64 (66.0%)	1367 (20-60 005)	
Prior chemotherapy	33 (34.0%)	1280 (20-21 640)	

Abbreviation: DLBCL, diffuse large B-cell lymphoma.
[a]Kruskal-Wallis 1-way analysis of variance.
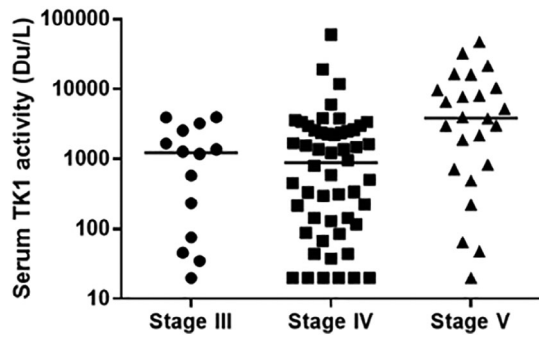[b]Mann-Whitney test.

FIGURE 1 Serum thymidine kinase 1 activity measured before treatment according to WHO clinical stage: stage III (n = 14), stage IV (n = 51), stage V (n = 24), Kruskal-Wallis 1-way analysis of variance, P = .02. The horizontal bars represent median
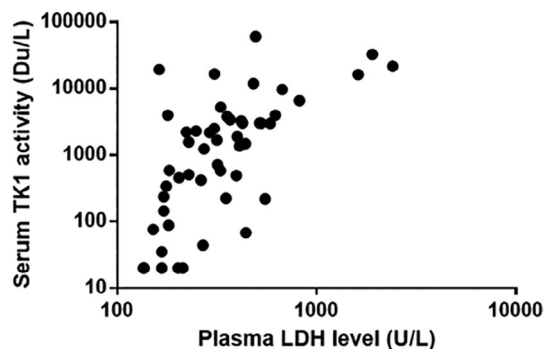

FIGURE 2 Serum thymidine kinase 1 (TK1) activity versus plasma LDH activity before treatment (n = 52). Serum TK1 activity showed a significant, strong, positive correlation with plasma LDH activity (r = .633, P < .0001). LDH, lactate dehydrogenase

FIGURE 3 Serum thymidine kinase 1 (sTK1) activity in healthy dogs (n = 23) and in dogs with complete (CR, n = 36) and partial (PR, n = 29) response. A, Serum TK1 activity distribution (Du/L) in healthy dogs and in dogs with complete versus partial response. The horizontal bars represent median. B, Receiver operating characteristic (ROC) curve of sTK1 activity assay for discrimination of complete versus partial response (AUC, 0.90; 95% CI, 0.816-0.985; P < .0001). Cutoff, 119.5 Du/L (sensitivity: 76%, specificity: 100%). AUC, area under the curve; CI, confidence interval; CR, complete response; NS, not significant; PR, partial response

respectively (Figure 3B). No healthy dogs had an sTK1 activity above 119.5 Du/L.

3.5 | Serum TK1 activity in the monitoring of remission in dogs with pretreatment sTK1 activity >20 Du/L

Thirty-six (36/65, 55%) treated dogs with pretreatment sTK1 activity above 20 Du/L went into complete remission and had sTK1 activity measured at 4-week intervals during follow-up. The median number of sTK1 activity samples per dog was 6 (range, 3-12). Seventeen of these 36 dogs (47%) remained in complete remission until the end of the study based on clinical examination, with a median follow-up of 113 days (range, 21-245 days). Nineteen of 36 (53%) dogs had a clinical relapse with a median follow-up of 122 days (range, 69-287 days). In dogs with clinical relapse during the follow-up, sTK1 activity evaluated 4 weeks before, and at the time of relapse was significantly higher than sTK1 activity measured at CR (Figure 4A). Serum TK1 activity measured at the time of relapse was not significantly different from pretreatment sTK1 activity (3014 versus 2207 Du/L; n = 19; P = .31). In dogs with persistent CR, median sTK1 activities measured at CR, 8 weeks before, 4 weeks before, and at last clinical evaluation
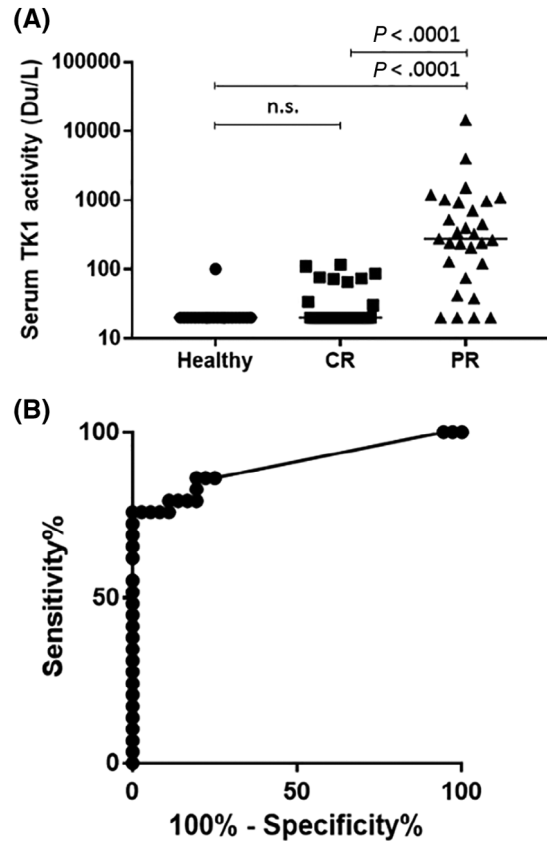
were not significantly different (Figure 4B). A 5-fold increase in sTK1 activity at a 4-week interval predicted relapse at the subsequent 4-week assessment with a sensitivity of 50% and a specificity of 94% (AUC, 0.72; 95% CI, 0.55-0.90; P = .02; Figure 5). An increase of the sTK1 activity (>2.7-fold the value measured at best clinical response) predicted relapse at the subsequent 4-week assessment with a sensitivity of 61% and a specificity of 88% (AUC, 0.79; 95% CI, 0.64-0.95; P = .004; Figure 6).

3.6 | Serum TK1 activity monitoring in dogs with pretreatment sTK1 activity ≤20 Du/L

Before any treatment, 12 of 97 (12%) dogs presented with an sTK1 activity ≤20 Du/L. Epidemiologic characteristics are summarized in Table S4. Many of these dogs were diagnosed with intermediate-grade lymphoma (5/12, 42%, dogs were diagnosed with small to medium cell lymphomas) or with a T-cell lymphoma (5/12, 42%), or
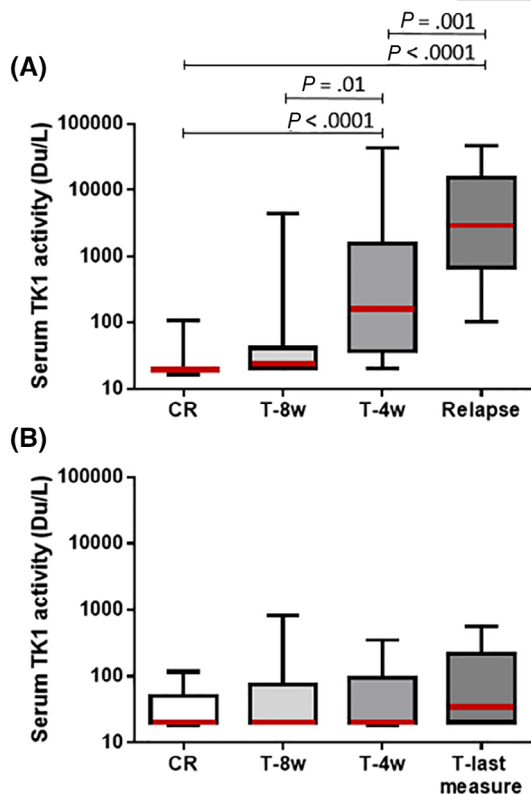
FIGURE 4 Serum thymidine kinase 1 (sTK1) activity in dogs with complete response and pretreatment sTK1 activity >20 Du/L. A, Serum TK1 activity distribution in dogs with complete response until clinical relapse (n = 19). Relapse, time of relapse. The red horizontal bars represent median. B, Serum TK1 activity distribution in dogs with complete response followed in persistent CR (n = 17). CR, time of complete response; T-4w, time 4 weeks before last clinical evaluation; T-8w, time 8 weeks before last clinical evaluation; T-last measure, time at last clinical evaluation. The red horizontal bars represent median

FIGURE 5 Serum thymidine kinase 1 (sTK1) activity ratio between activity measured 4 weeks (T-4w) and 8 weeks (T-8w) before relapse or last clinical evaluation in dogs with relapse (n = 19) versus persistent CR (n = 17). A, Serum TK1 T-4w/T-8w activity distribution between relapse versus persistent CR. The horizontal bars represent median. B, Receiver operating characteristic (ROC) curve of sTK1 T-4w/T-8w for detecting clinical relapse within the 4 following weeks in dogs with complete response (AUC, 0.72; 95% CI, 0.55-0.90; P = .02). Cutoff, 5 (sensitivity: 50%, specificity: 94%). AUC, area under the curve; CI, confidence interval; CR, time of complete response; T-4w, time 4 weeks before last clinical evaluation; T-8w, time 8 weeks before last clinical evaluation

received prior chemotherapy before inclusion (4/12, 33%). The 2 dogs diagnosed with DLBCL with pretreatment sTK1 activity ≤20 Du/L had received prior chemotherapy. Eleven of 12 (92%) dogs received chemotherapy after enrollment (5 dogs received a 12-week free maintenance CHOP-based protocol and 6 dogs received a single-agent protocol). All dogs had an objective clinical response (5 CR and 6 PR), and sTK1 activity remained ≤20 Du/L at the time of the best clinical response. Eight of 11 (73%) dogs had clinical relapse during the follow-up. In these 8 dogs, median sTK1 activity 4 weeks before and at the time of relapse was 305 Du/L (range, 20-1684 Du/L) and 350 Du/L (range, 74-12 113 Du/L), respectively, and was significantly higher than sTK1 activity measured before treatment and at the time of their best clinical response (Figure 7).

4 | DISCUSSION

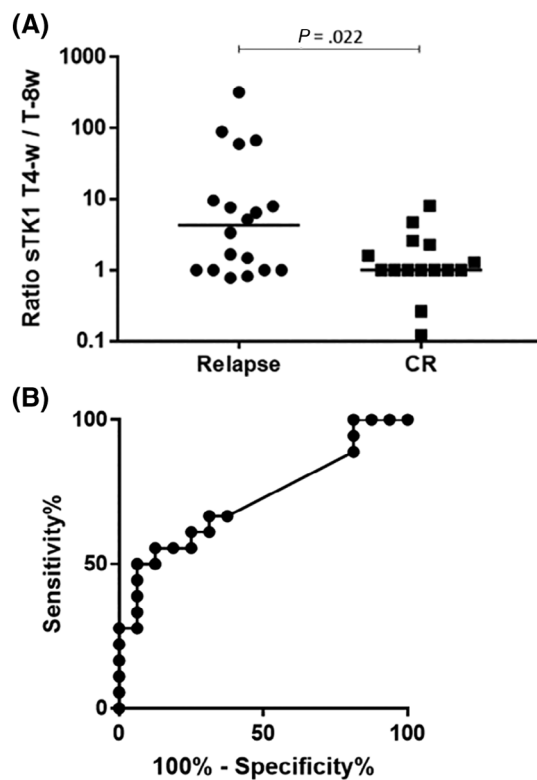This prospective descriptive study demonstrates that sTK1 activity is a blood marker potentially useful to evaluate treatment effectiveness and to predict relapse before clinically detectable disease in dogs with intermediate to high-grade NHL. Serum TK1 activity was significantly higher in dogs with lymphoma compared to healthy dogs. After treatment initiation, sTK1 activity was significantly lower in dogs that fully responded to treatment and an increase of sTK1 activity was predictive for relapse after CR. Blood biomarkers are of great interest in human and veterinary oncology as noninvasive tools for assessing treatment response and disease monitoring. Interest in TK1 as a tumor biomarker began in the 1970s and increased more recently with the development of nonradiometric enzymatic assay which has its largest clinical implication in hematologic neoplasia.[53-55,67-71]

In this study, sTK1 activity was ≤20 Du/L in 22 of 23 (96%) dogs from the healthy control group. For the purpose of comparison, we decided to consider sTK1 activity increased when the sTK1 activity was above 20 Du/L and dogs with NHL were analyzed in 2 groups based on pretreatment sTK1 activity level (≤20 versus >20 Du/L). Only 1 healthy dog had the sTK1 activity above 20 Du/L with an
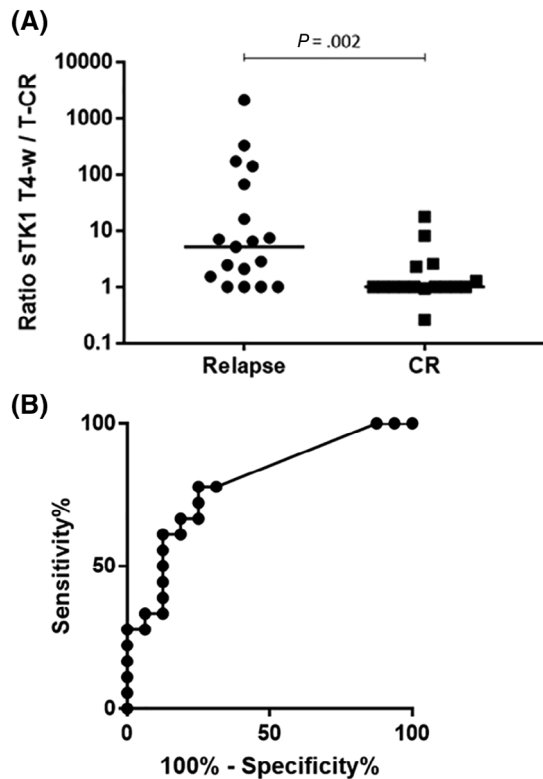
FIGURE 6 Serum thymidine kinase 1 (sTK1) activity ratio between activity measured 4 weeks (T-4w) before relapse or last clinical evaluation and at the time of complete response (T-CR) in dogs with relapse (n = 19) versus persistent CR (n = 17). A, Serum TK1 T-4w/T-CR activity distribution between relapse versus persistent CR. The horizontal bars represent median. B, Receiver operating characteristic (ROC) curve of sTK1 T-4w/T-CR for detecting clinical relapse within the 4 following weeks in dogs with complete response (AUC, 0.79; 95% CI, 0.64-0.95; P = .004). Cutoff, 2.7 (sensitivity: 61%, specificity: 88%). AUC, area under the curve; CI, confidence interval
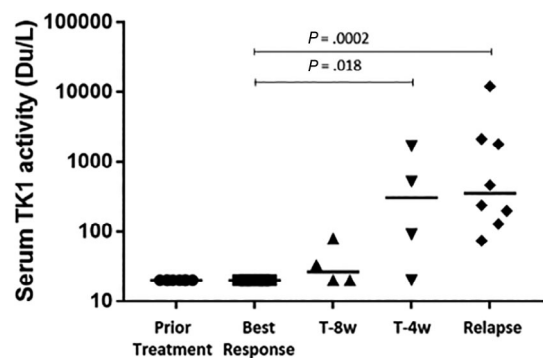
FIGURE 7 Serum thymidine kinase 1 (sTK1) activity monitoring in dogs with pretreatment sTK1 activity ≤20 Du/L followed until relapse (n = 8). CR, time at best clinical response; T-4w, time 4 weeks before relapse; T-8w, time 8 weeks before relapse. The horizontal bars represent median

sTK1 activity measured at 101 Du/L. This dog was considered healthy based on the physical examination with no history of clinical signs for a minimum of 6 months before inclusion. However, no further investigations were performed on this dog, and an asymptomatic disease

was not excluded. In dogs with naturally occurring intermediate- to high-grade NHL, 85 of 97 (88%) dogs had sTK1 activity above 20 Du/L before any treatment. Published studies in dogs with multicentric lymphoma described an increased pretreatment sTK1 activity in 47% to 100% of dogs.[49,53-55] However, most dogs in these studies did not have tumor histologic grade, immunophenotype, clinical stage, and cytomorphological subtype systematically determined that might explain the discrepancy of these results. In our study, pretreatment sTK1 activity increased with more advanced clinical stage and was significantly higher in dogs with confirmed stage V lymphoma. Pretreatment sTK1 activity was significantly higher in dogs with B-cell lymphoma and in dogs with WHO substage b, confirming previously published results.[53,55] As in humans, several subtypes of canine NHLs have been associated with significant differences in biological features and clinical behavior. In our study, dogs with DLBCL had higher pretreatment sTK1 activity than dogs with other cytomorphological subtypes. A significant correlation was reported between LDH activity and TK1 activity before any treatment in 20 dogs with lymphoma and leukemia.[54] This finding had, however, not been supported by another larger study.[72] In our present study, even if LDH activity was significantly correlated with sTK1 activity, only 33% (17/52) of dogs had increased pretreatment LDH activity level. Our results suggest that LDH activity should not be used as a specific biomarker for NHL.

In 65 treated dogs with pretreatment sTK1 activity >20 Du/L, a significant decline in sTK1 activity was observed with objective tumor response. In dogs with CR, sTK1 activity was not significantly different from the sTK1 activity in healthy dogs. Serum TK1 activity showed an interesting sensitivity of 76% and an excellent specificity of 100% to discriminate dogs with CR versus PR. These results are in concordance with previous studies suggesting that in treated dogs with increased sTK1 activity at diagnosis, sTK1 activity drops to minimal level when the clinical remission has been achieved.[49,53-55,73] One limitation in this study is that dogs were not completely restaged at the time of their best clinical response and sTK1 activity measurement to confirm the CR status. In 9 of 36 (25%) dogs classified as being in complete remission, the sTK1 activity remained above 20 Du/L at the time of their best response. No further investigations have been performed on these dogs to confirm the CR status because blood samples were run in batches and sTK1 activity results were obtained a posteriori.

In our study, sTK1 activity was also predictive for relapse after CR. Monitoring sTK1 activity allowed identification of dogs in remission with subclinical progressive disease that developed relapse 4 weeks later. Of 36 dogs in CR that had pretreatment sTK1 activity >20 Du/L, a 5-fold increase in sTK1 activity at a 4-week interval or an increase by >2.7-fold the value measured at best clinical response predicted relapse at the subsequent 4-week assessment, with a sensitivity of 50% and 61% and a specificity of 94% and 88%, respectively. These results suggest likely clinical benefit of sTK1 activity in the monitoring of the remission status with a simple blood sample performed every 4 weeks after completion of the free maintenance chemotherapy protocol. The lack of sensitivity for detecting dogs that developed a relapse might be explained by the rapid progression of

the disease. All dogs in this study presented with a significant increase of sTK1 activity at the time of relapse. However, in 8 of 19 (42%) dogs with clinical relapse during the follow-up, the sTK1 activity remained ≤20 Du/L 4 weeks before relapse. For these dogs, an increase of sTK1 activity closer to the time of detectable progressive disease is suspected and additional blood samples, every week or every other week, would be necessary to more accurately capture the sTK1 increase in subclinical progressive disease. Serum TK1 activity revealed an interesting specificity (94% and 88%) to predict relapse 4 weeks before clinically detectable disease. However, a few dogs had a transient increase in sTK1 activity during persistent clinical complete remission; the precise explanation for this remains unknown. Five of 17 (29%) dogs, monitored in persistent complete remission until the end of the study, had their sTK1 activity above 20 Du/L during the follow-up. Most of these dogs (4/5, 80%) presented with a confirmed stage V disease at inclusion. Transient increase of serum TK1 activity might be expected when neoplastic cells died after treatment initiation, releasing TK1 into the blood. Even though, dogs were considered in remission based on physical examination, persistent internal/residual disease (bone marrow infiltration) could not be excluded as a source of persistent sTK1 activity. In this context, further investigations (repeated imaging, cytology) might be indicated to suspect progressive disease in treated dogs with increased sTK1 activity as false-positive results could lead to unnecessary adaptation of the chemotherapy protocol.

Twelve of 97 (12%) dogs with NHL presented with a low sTK1 activity (≤20 Du/L) before treatment and sTK1 activity remained low (≤20 Du/L) at the time of their best clinical response. Two dogs diagnosed with DLBCL had pretreatment sTK1 activity ≤20 Du/L. These 2 dogs received prior chemotherapy that might explain the low activity level at inclusion. Eight of 12 (67%) dogs experienced a clinical relapse during the follow-up. In these 8 dogs, median sTK1 activity 4 weeks before (measured in 4 dogs) and at the time of relapse (measured in 8 dogs) was significantly higher than sTK1 activity measured before treatment and at the time of their best clinical response. This significant finding suggests that sTK1 measurement might be useful in the monitoring of the remission status in these patients.

As dogs received a nonstandardized chemotherapy protocol in our study, correlation with disease-free interval (DFI) or survival time was not considered relevant by the authors. Therefore, sTK1 activity was not evaluated as a prognostic factor in duration of remission and survival. Association of pretreatment sTK1 activity with clinical outcome has been previously evaluated in dogs with NHL. Pretreatment sTK activity was reported to be correlated with survival time.[53] However, these results were not confirmed in a larger study, indicating that initial sTK1 activity might not be such a good potent prognostic indicator of DFI or survival in dogs with NHL.[55] Nevertheless, future studies should be considered to analyze the prognostic significance of sTK1 activity at the end of chemotherapy. We hypothesize that increased sTK1 activity after discontinuation of the chemotherapy could correlate with shorter duration of remission and survival.

The present study has several limitations, including population size and, potentially, the heterogeneity of the lymphoma population.

Particularly, T-cell lymphoma was associated with a lower sTK1 activity compared to B-cell lymphoma. In our study, 15 of 97 (15%) dogs were diagnosed with a T-cell lymphoma, but this finding is in concordance with previous studies reporting a proportion of T-cell lymphoma from 13% to 32%.[19,21,23,27,74] Dogs with various types of lymphoma including stage, substage, immunophenotype, and morphological subtype might be associated with different sTK1 activity profile. However, that significant predictive ability was found here in a heterogenous population and could allow wider application of the results within several types of NHL. Another limit as previously discussed is that complete clinical staging was not performed at the time of repeated sTK1 analysis to confirm CR status as sTK1 activity measurement was performed a posteriori to the clinical monitoring.

Finally, previous studies showed that sample handling and transport conditions might affect sTK1 activity results.[75] Serum TK1 activity has previously been reported to be stable when blood samples were centrifuged and serum removed within 1 hour and stored at −20°C. Therefore, results in this present study would not be expected to have been significantly affected by freezing and storage before analyzing samples.

Earlier techniques for measuring serum TK1 activity used a radioenzyme-based assay (TK-REA).[53,54] The TK-REA is a radioenzymatic assay using ^{125}I-iododeoxyuridine ($[^{125}$I]-dUrd), which is converted by TK1 to $[^{125}$I]-dUrd monophosphate ($[^{125}$I]-dUMP). It has been used for several decades and is an established tool for tumor diagnosis and prognosis in human medicine and in dogs with lymphoma.[53,70] However, the use of the radioactive enzyme substrate limited wide application in clinical practice because of cost, safety issues, and equipment required to perform the test. Recently, new nonradioactive assays for the measurement of TK1 activity in serum have been developed using enzyme-labeled immunoassay (TK-ELISA).[49,72,76] The TK-ELISA assay has been tested in sera from dogs with lymphoma and provided useful information for monitoring treatment and predicting relapse of the disease.[49,72] Nonradiometric TK1 assay was recently validated against the TK-REA in dogs and studies showed a high correlation with the TK-REA.[76] In the present study, the serum TK1 activity was determined by a new refined ELISA-based method, the DiviTum assay (Biovica International). The DiviTum assay showed a high correlation with the TK-ELISA assay (Liaison assay; DiaSorin, Stillwater, Minnesota) in serum of human breast cancer patients (r = .90) with concordance of 91.3%.[60] In humans, preoperative measurements of TK1 activity with the DiviTum assay provide prognostic information in primary breast, lung, and renal cancers.[64,77,78] The DiviTum assay provides valuable clinical information in the treatment and management of chronic lymphocytic leukemia.[62] Consequently, the DiviTum method is valuable to encourage further investigation in veterinary patients.

In conclusion, the monitoring of sTK1 activity as a tumor biomarker in veterinary medicine is promising. Serum TK1 activity demonstrated strong abilities to detect complete clinical response and subclinical progressive disease before clinical relapse in treated dogs with NHL. Serum TK1 activity might be particularly interesting in dogs with DLBCL, associated with an increased activity at diagnosis, as an

increase of sTK1 activity during the follow-up period might help identify dogs with high risk of relapse. Therefore, with a simple blood sample, it could be possible to monitor remission status and suspect relapse 4 weeks before it becomes clinically evident.

CONFLICT OF INTEREST DECLARATION

Biovica International provided support in the form of salaries for M. Bergqvist and G. Sack. G. Sack is a former employed by Biovica International. M. Bergqvist and G. Sack hold shares in Biovica International. Biovica International did not have any additional role in the study design, data collection, decision to publish, or preparation of the manuscript. Oncovet-Clinical-Research (OCR) provided support in the form of salaries for authors F. Floch and D. Tierny. Oncovet-Clinical-Research did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. Soladis Clinical Studies is a partner of OCR. No potential conflicts of interest were disclosed by the other authors.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

This study was approved by the OCR Ethical Committee.

HUMAN ETHICS APPROVAL DECLARATION

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

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1738 | Journal of Veterinary Internal Medicine

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