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Serum TK1 protein and C-reactive protein correlate to treatment response and predict survival in dogs with hematologic malignancies

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ABSTRACT

Thymidine kinase 1 (TK1), involved in DNA precursor synthesis, is used as a serum biomarker in cancer diagnostics in both human and veterinary medicine. We investigated the utility of serum TK1 protein (TK1p) and TK1 activity (TK1a) determinations for prognosis and monitoring of canine hematological malignancies. The combination of TK1p or TK1a with canine C-reactive protein (CRP) determinations was also investigated.

Serum samples from 51 client-owned dogs with naive hematological malignancies and from 149 healthy subjects were included. Serum TK1p levels were determined using a prototype TK1-ELISA, TK1a using the [^{3}H]–dThd phosphorylation assay, and CRP using an immunoturbidimetric assay.

Mean TK1p in sera from dogs with tumors was significantly higher than from healthy dogs (mean ± SD = 3.9 ± 5.9 vs. 0.45 ± 0.15 ng/mL). Similarly, TK1a in hematological malignancies was significantly higher than in healthy dogs (mean ± SD = 15.1 ± 31.3 vs. 0.96 ± 0.33 pmol/min/mL). The receiver-operating characteristic indicated that a combination of TK1p or TK1a with CRP gave higher sensitivity than either biomarker alone for the prognosis of hematological malignancies. Median pretreatment TK1p and TK1a levels were significantly higher than in dogs in remission and correlated with clinical outcome. Kaplan-Meier curve analysis showed that naive dogs with high TK1p, TK1a, and CRP had significantly shorter survival.

This study present two new polyclonal antibodies used in an ELISA system to determine TK1p. The study also show that combining TK1p or TK1a with CRP gave higher sensitivity than either biomarker alone. Monitoring patients in the study while undergoing chemotherapy, suggests that the TK1 + CRP combination could be useful in a biomarker panel, possibly aiding the prognosis and therapy monitoring of hematological malignancies in dogs.

1. Introduction

Malignant lymphomas are among the most common aggressive tumors affecting dogs (Ettinger, 2003; Teske, 1994; Vail and Young, 2007) with an estimated annual incidence of 25–40 per 100,000 dogs and accounting for approximately 5% of all canine malignancies (MacEven, 1990; Teske, 1994). There are many similarities between canine lymphoma and human non-Hodgkin's lymphoma (NHL) such as clinical presentation, molecular biology, treatment, and treatment response (Teske, 1994; Vail and MacEwen, 2000). This makes canine lymphoma in client-owned dogs with spontaneous occurring disease a valuable comparative model for human NHL. The search for biomarkers that are elevated in the blood before clinical symptoms of cancer appear has been intensive in both human and veterinary oncology (Hallek et al., 1992; O'Neill et al., 2001). Several biomarkers of canine lymphomas, such as alpha 1-acid glycoprotein (Hahn et al., 1999b), glutathione-S-

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transferase (Hahn et al., 1999a), and lactate dehydrogenase (Marconato et al., 2010), increase in canine lymphomas before relapse, though they were found to be of minimal prognostic value, and the data on alpha-fetoprotein levels are conflicting (Hahn et al., 1999a).

Thymidine kinase 1 (TK1) is involved in the salvage pathway for DNA precursor synthesis and is expressed preferentially in the S-phase of the cell cycle. Due to the accelerated and dysregulated cell proliferation during cancer progression, both TK1 activity (TK1a) and TK1 protein (TK1p) levels increase markedly, and TK1 is released into the bloodstream presumably by disruption of the dividing tumor cells (Gronowitz et al., 1984; Jagarlamudi et al., 2014). In dogs with lymphoma, high TK1 levels are correlated with advanced disease stage and poor prognosis (Nakamura et al., 1997; von Euler et al., 2004; von Euler et al., 2009; vonEuler and Eriksson, 2011). These older studies referred mostly to TK1a measurements, but more recent studies have described changes in TK1p concentration, which also seemed to be correlated to prognosis (Jagarlamudi et al., 2014; Kiran Kumar et al., 2013). Unlike TK1a, TK1p determinations do not require a fully functional protein. There are several methods to determine TK1a; the classical one relies on the phosphorylation of ^{3}H-Thymidine to ^{3}H-Thymidine monophosphate using ATP as a phosphate donor more recently optimized by Sharif et al. (Sharif et al., 2012). This method is highly correlated to other TK1a methods such as TK REA (Gronowith et al., 1984), TK Liaison (von Euler et al., 2009), and Divitum (Boye et al., 2019). The association between tumor development and inflammation plays an important role in tumor cell survival (Orr et al., 2011). Among the most commonly used inflammatory markers in routine diagnostics is C-reactive protein (CRP).

CRP increase in tumors is likely a product of tissue damage caused by the tumor, which often promotes the inflammation cascade. Low-grade inflammation predisposes the subject to cancer development. This has been proven in many forms of tumor diseases in human (Asegaonkar et al., 2015; Fang et al., 2015) and veterinary medicine where CRP has been used to predict prognosis and to monitor cancer treatments (Alexandrakis et al., 2017; Fontaine et al., 2017; Merlo et al., 2007; Mischke et al., 2007; Nielsen et al., 2007).

TK1a and serum canine-specific C-reactive protein (c-CRP) have been used as a neoplastic index (NI) in screening healthy dogs for occult cancer (Selting et al., 2016) and the combination of TK1a and CRP levels significantly increased the diagnostic accuracy. The NI could therefore be used to differentiate dogs at high risk of cancer development from dogs at low risk (Selting et al., 2015).

The aims of this study were to measure TK1p using a new prototype TK1-ELISA based on two different polyclonal antibodies against selected epitopes on canine TK1. These were used to develop an ELISA to measure TKp.TK1a and CRP were also measured in sera from dogs with hematological malignancies, and to evaluate the prognostic value of combining these biomarkers. Other known clinical parameters that may have prognostic relevance were also considered. In addition, the utility of TK1 and CRP in predicting overall survival and monitoring relapse in canine lymphoma patients during chemotherapy was investigated.

2. Materials and methods

2.1. Patients and diagnosis of lymphoma

The dogs in the hematological malignancy group ($n = 51$) were newly diagnosed with hematological malignancies, primarily lymphoma, and presented at the University Animal Hospital at the Swedish University of Agricultural Sciences (SLU) Uppsala, Sweden. The diagnosis of lymphoma or leukemia was based on cytology ($n = 31$) or histopathology ($n = 20$) combined with physical examination and clinical presentation. Full clinical staging and typing of lymphomas were not performed for all patients primarily for financial reasons. Data covering blood count, biochemistry panel, and urinalysis were gathered for all dogs. The dogs with tumors were naive and had not received any prior antitumor treatment for cancer.

The TK1p and TK1a levels were followed during chemotherapy for twenty of the dogs and in some dogs also their CRP levels were sequentially followed. One dog had been treated with prednisolone before referral to the University Animal Hospital and was therefore not included among the 51 naive dogs.

The group of 149 healthy dogs was considered healthy based on medical history, physical examination, hematology, and a basic biochemistry analysis. These subjects were mainly recruited from the group of voluntary blood donor dogs at the University Animal Hospital.

2.2. Serum samples and specimen handling

Serum samples from dogs with naive hematological malignancies and from healthy dogs were collected over a 4-year period (2015–2019). At least 1 mL of blood was drawn from each patient and centrifuged within 1 h of collection. The serum samples were stored at −20 °C until analysis. This project was approved by the Swedish Animal Ethics Committee (ref. no. C12/15) and samples were used only with the owners' signed consent. The dogs were sampled on their first visit to the oncology service and thereafter on follow-up visits as decided by the treating veterinarian, mostly depending on the chemotherapy administration protocol.

2.3. Chemotherapy treatment

Patient dogs were treated according to a modified L-CHOP protocol (Piek et al., 1999). All cases that relapsed or did not respond were further subjected to a modified rescue protocol based on lomustine (70 mg m^{-2} once every 3 weeks) and/or prednisolone as described (Moore et al., 1999). Some owners declined chemotherapy and all except one of their dogs were treated with prednisolone as the single agent.

2.4. Dog Anti-TK1 polyclonal antibodies and recombinant dog TK1

The dog TK1 ELISA was developed by using two polyclonal antibodies that were raised against the different regions of TK1. The antisera were produced by GenScript (Piscataway, NJ, USA) using 16-aminoacid synthetic peptide (PAb1-CVLVPGKPGEGKEATG, aa211–225) and one 24-aminoacid synthetic peptide (PAb2- AYTKRLGSEKEVE-VIGGADKYHSC, aa161–183) as antigens. The antisera were collected from the rabbits after the 3rd and 4th immunizations and purified on two TK1 peptide coupled Sepharose 4B columns respectively, as described previously. High levels of inactive thymidine kinase 1 polypeptide detected in sera from dogs with solid tumors by immunoaffinity methods: implications for in vitro diagnostics (Kiran Kumar et al., 2013). The recombinant dog TK1 was cloned and expressed in E.Coli and purified by Ni- Sepherose affinity chromatography as previously described (Hanan et al., 2012). Different concentrations of recombinant dog TK1 was used to prepare the calibrators. The antibodies were supplied by Alertix Veterinary Diagnostics AB (Kalmar, Sweden).

2.5. Dual polyclonal canine TK1-ELISA (dual Pab ELISA)

The ELISA was performed as described previously (Jagarlamudi et al., 2015). In brief, the ELISA plates (NUNC Maxisorp, Thermo Fisher Scientific, Waltham, MA, USA) were coated with the anti-dog TK1 antibody (PAb1; 4 μg/mL) in 100 mM of carbonate buffer (pH 9.6) and incubated overnight at 4 °C. The plates were then washed four times with wash buffer (Tris-HCl, 0.1 M, pH 7.6; NaCl, 0.3 M; Tween 20, 0.1%; and BSA, 0.5%) and blocked with non-fat dry milk 5% in TBST (tris-buffered saline and Tween 20) for 1 h at room temperature (RT). Dog serum samples (80 μL) were diluted 1:1 with a sample dilution buffer (Alertix Veterinary Diagnostics AB, Kalmar, Sweden) along with recombinant dog TK1 in concentrations ranging from 0 to 10 ng/mL, which served as calibrators. Both samples and calibrators were pre-incubated for 1 h at RT. The coated plates were washed four times

with wash buffer; 100 µL of diluted recombinant TK1 and serum samples were added to each well, and the plates were incubated for 2 h.

The plates were then washed four times and incubated with a biotin-labeled anti-dog TK1 antibody (PAb2; 3 µg/mL) for 1 h at RT. The plates were washed again and incubated with 100 µL of streptavidin-HRP (Thermo Fisher Scientific, Sweden) for 30 min. After a final wash, the plates were incubated with 100 µL of TMB (3,3′,5,5′-Tetramethylbenzidine) for 15 min in the dark. The reactions were stopped by adding 100 µL of 1 N HCL and the absorbance was measured at 450 nm with a micro reader (Sunrise Reader, Tecan Life Science, Zurich, Switzerland). The limit of blank (LOB) is 0.06 ng/mL and the lower limit of detection (LOD) is 0.11 ng/mL. All samples were run in duplicate, and the results were analyzed by means of linear regression using the standard calibrators and GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). The TK1p concentrations in the serum samples were expressed in ng/mL. The sample dilution buffer was used as a blank (concentration 0). The cut-off value was determined as mean + 2 SD of the serum TK1 concentrations of the 149 healthy dogs. The median inter- and intra-assay variations were 12% and 10%, respectively.

2.6. [^{3}H]-dThd phosphorylation assay

The TK1a was measured by means of radiochemical assay using the DE-81 filter paper technique as described previously (Sharif et al., 2012). The reaction mixture contained Tris–HCl (pH 7.6, 10 mM), dithiotreitol DTT (2 mM), $MgCl_2$ (5 mM), NaF (5 mM), ATP (5 mM), [^{3}H]-dThd (5 µM), and 10 µL of serum to a final volume of 40 µL. All serum samples were analyzed in triplicate and the cut-off value was established based on 149 healthy dogs (Sharif et al., 2012). The detection limit (LOD) based on ten runs of samples with very low TK1 activity is 0.34 pmol/min/mL and the limit of quantification (LOQ) is 0.9 pmol/min/mL.

2.7. Canine CRP assay

Subgroup sera from healthy dogs (n = 30) and from dogs with hematological malignancies (n = 38) were analyzed for their CRP levels using an immunoturbidimetric, canine-specific CRP reagent (Gentian AS, Moss, Norway) in an automated, open-system clinical chemistry/immunoassay analyzer (Abbott Architect c4000, Abbott Park, IL, USA). The samples were analyzed at the Clinical Pathology Laboratory, SLU, Uppsala, Sweden. The cut-off value was estimated based on sera from the healthy dogs (n = 30). The analytical performances of the assay in terms of inter- and intra-assay variations were described previously (Hillstrom et al., 2014).

2.8. Statistical analysis

Distributions of TK1p and TK1a levels in healthy and hematological cancer sera were evaluated for normality using the D'Agostino and Pearson omnibus normality test. The TK1p in sera from heathy dogs showed Gaussian distribution but the TK1a levels displayed non-Gaussian distribution, and the Mann-Whitney U test was used for comparison between groups. Receiver-operating characteristic (ROC) curves were constructed to evaluate the performance of the dog TK1-ELISA, TK1a, and a combination of these with CRP. All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software). Both combinational ROC curves and median survival were calculated with the Kaplan-Meier curve using MedCalc version 17.6 (MedCalc Software, Ostend, Belgium) statistical discovery software. A factor with a P-value <0.05 was considered significant. Dogs were censored if they were still alive at manuscript submission or lost to follow-up.

3. Results

3.1. Study population

The dogs in the hematological malignancy group (n = 51), which were 2–14.8 years old with a median age of 7.9 years, comprised 19 males, 20 females, 7 neutered males, and 5 spayed females. For further epidemiological and clinical characteristics of the dogs in the hematological malignancy and healthy control groups, see Tables 1 and 2. The dogs in the healthy control group (n = 149), which were 1–6.9 years old with a median age of 3 years, comprised 88 males, 46 females, 13 neutered males, and 2 spayed females. The dogs in the hematological malignancy group were older than the healthy subjects ($P \leq 0.0001$), with median ages of 7.9 and 3 years, respectively.

3.2. TK1 protein and TK1 activity levels in serum samples

Healthy dogs. TK1p and TK1a levels were determined in sera from 149 healthy dogs. The TK1 concentration in the serum samples was determined using a recombinant canine TK1 standard curve (Fig. 1A). The TK1p concentrations in healthy subjects were normally distributed, ranging from 0.17 to 0.96 ng/mL (median = 0.45), as shown in Fig. 1B. The TK1a levels in healthy subjects were not normally distributed, ranging from 0.34 to 1.9 pmol/min/mL (median = 0.96), as shown in Fig. 1C. The cut-off value for healthy dogs were determined by using mean TK1 value of the group +2 * standard deviation. The cut-off value for TK1 protein is 0.75 ng/mL and for TK1 activity is 1.6 pmol/min/mL. These cut-off values were used to construct the ROC curves that provides overall assay performance in relation to hematological malignancies.

Table 1
Epidemiological characteristics of the dogs in the hematological malignancy and healthy control groups.

Epidemiological characteristics	Hematological malignancy group	Healthy control group
Sex	Female 20 Female, spayed 5 Male 19 Male, neutered 7	Female 46 Female, spayed 2 Male 88 Male, neutered 13
Age median (range), *years*	7.9 (2–14.8)	3 (1–6.9)
Body weight median (range), *kg*	23.7 (4.5–64)	34 (26–56)
Breed	Mixed breed 12, Golden retriever 4, German shepherd 3, Nova Scotia duck tolling retriever 2, Jack Russell 2, Rottweiler 2, Dogue de Bordeaux 1, Norwegian elkhound 1, Swedish elkhound 1, Staffordshire bullterrier 1, Boxer 1, Danish–Swedish farm dog 1, Hovawart 1, Whippet 1, Great Dane 1, Siberian husky 1, Lagotto Romagnolo 1, Hamilton hound 1, Perro de agua d'Espagnole 1, Yorkshire terrier 1, Border collie 1, Chihuahua 1, Labrador 1, Pug 2, Welsh springer spaniel 1, Miniature schnauzer 1, Chinese crested powder puff 1, Dachshund 1, Riesenschnauzer 1, Scottish terrier 1	Mixed breed 30, Flat-coated retriever 20, German shepherd 20, Labrador 16, Golden retriever 13, Greenland dog 9, Dobermann 7, Boxer 5, Australian kelpie 4, Saluki 4, Riesenschnauzer 4, Cane corso 3, Irish setter 3, Collie (long haired) 2, Greyhound 2, Bernese mountain dog 1, Beauceron 1, Rhodesian ridgeback 1, Bracco italiano 1, White Swiss shepherd 1

Table 2
Clinical characteristics of the hematological malignancy group.

Clinical characteristics	Hematological malignancy group
WHO sub-stage	A 22 B 26
Type of B-symptoms	lethargy 20, weight loss 9, GI symptoms 9, fever 3, PU/PD 3, retinal bleeding 2, neurological symptoms 1, abdominal pain 1
Suspected tumor cells in peripheral blood	Yes 10 No 41
Diagnosis by	Histology 20 Cytology 31
Grade	High 37 Intermediate grade 8 Low grade 2 Not specified 4
Hypercalcemia	4
Large granular cell lymphoma (LGL)	3
Cutaneous lymphoma	2
Myelocytic leukemia	1
Lymphatic leukemia	2
Indolent lymphoma	1
Treatment	No treatment (N) 11, L-asparaginase (L) 2, Prednisolone (P) 2, Doxophos (D) 1, L-asparaginase + prednisolone 2 (LP), Adriamycin-based protocol (A) 12, Adriamycin-based protocol + lomustine (AC) 3

3.3. Dogs with hematological malignancies

The TK1p concentrations in sera from dogs with hematological malignancies were 0.24–26.5 ng/mL (median = 1.4). There was a significant difference between the healthy dogs and dogs with hematological malignancies ($P \leq 0.0001$, Fig. 1B). Similarly, TK1a levels in dogs with hematological malignancies were 0.4–150 pmol min/mL (median = 3.6). Using the Mann-Whitney *U* test, the level of TK1a was significantly higher in the hematological malignancy group than in the healthy subjects ($P \leq 0.0001$, Fig. 1C). Of the 51 samples in the hematological malignancy group, 10 were placed in the low/intermediate-grade lymphoma subgroup and 37 in the high-grade lymphoma subgroup; four samples were not specified. Both TK1p and TK1a levels in the high-grade group were significantly higher than in the low/intermediate-grade group ($P = 0.0002$ and $P < 0.0001$, respectively) as shown in Fig. 2A and B. Furthermore, ROC curve analysis showed that TK1-ELISA had an area under the curve (AUC) of 0.89 ($P < 0.0001$ [95% confidence interval (CI), 0.83–0.95]) with a sensitivity of 0.70 and specificity of 0.95 at a cut-off value of 0.75 ng/mL which is based on healthy dog TK1 protein levels (Fig. 2C). The TK1a had a sensitivity of 0.78 and a specificity of 0.95 with an AUC of 0.87 ($P < 0.0001$ [95% CI 0.80–0.95]) at the optimal cut-off value of 1.6 pmol/min/mL based on healthy TK1 activity values (Fig. 2D). By means of linear regression analysis, a significant correlation was found between canine TK1-ELISA and the [^{3}H]-dThd phosphorylation assay in hematological malignancies ($r_s = 0.84$, $P \leq 0.0001$), but not in the healthy group ($r_s = 0.12$, $P = 0.13$). Two out of three large granular cell lymphomas in this study had clearly elevated TK1a (15 and 7,22 pmol min/mL), TK1p (5,33 and 1,13 ng/mL) and CRP (132 and 56,3 mg/L) values, CRP not shown in the figures.

3.4. Combination of TK1 and CRP

Subgroups of sera from healthy dogs and from those with hematological malignancies were also evaluated for their CRP levels. The baseline levels of CRP were established in 30 healthy dogs and in 36

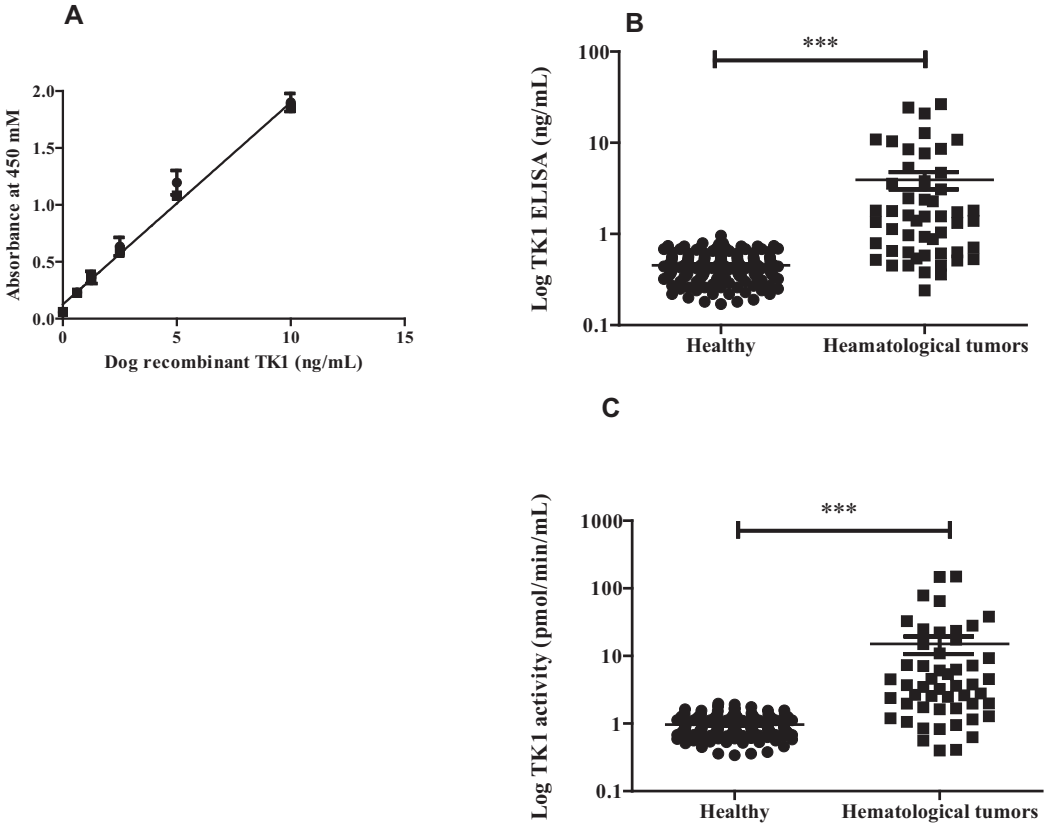

Fig. 1. (A) The standard curve for recombinant dog TK1 (0–10 ng/mL) measured using the dual PAb ELISA. Error bars represent the standard deviation of ten runs. (B) Log TK1p concentrations measured using the PAb TK1-ELISA in sera from healthy dogs ($n = 149$) and dogs with hematological malignancies ($n = 51$). (C) Log TK1a distribution with sera from healthy dogs ($n = 149$) and sera from dogs with cancer ($n = 51$). The error bars represent the median values.

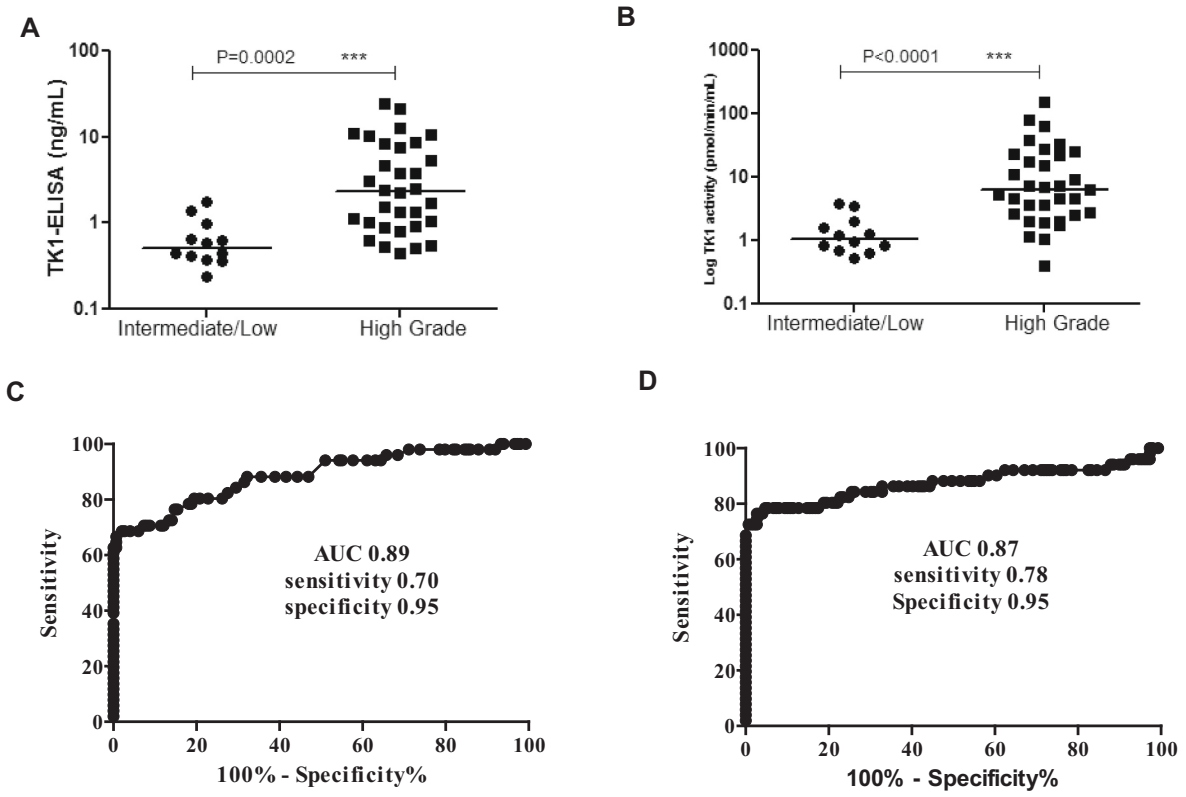

Fig. 2. Grading of hematological malignancies based on cytology in 47 out of 51 serum samples. TK1p (A) and TK1a (B) in relation to low/intermediate ($n = 10$) and high-grade ($n = 37$) subgroups. Receiver-operating characteristic (ROC) curve of the results with TK1-ELISA using sera from dogs with hematological malignancies versus sera from healthy dogs (C). A similar ROC curve analysis of the TK1a assay results with sera from healthy dogs and dogs with hematological malignancies (D).

dogs with hematologic malignancies. The median CRP level in healthy dogs was 4.42 mg/L (range: 2.88–9.60), while in the hematological malignancy group it was 19 mg/L (range: ≤7–163). By using binary logistic regression, we determined a neoplastic index (NI) using TK1-ELISA and CRP values as well as TK1a and CRP, analogous to the NI described by (Selting et al., 2015).

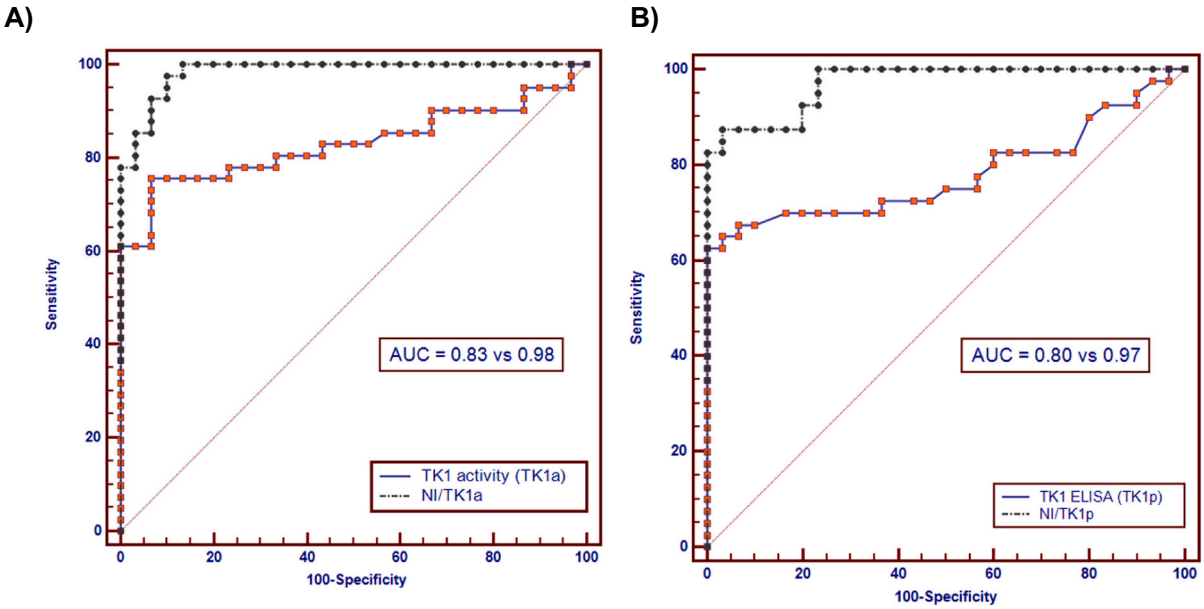

Fig. 3. A The logarithmic of the combination of TK1-ELISA and TK1 activity with CRP. Subgroups of 30 samples from healthy dogs and 36 samples from dogs with hematological malignancies were analyzed in the three assays. The ROC curve was set up by combining (A) TK1p and CRP, the red dot with blue line indicates the TK1p assay and blue dots with black line indicates the combination of TK1p and CRP. (B) TK1a and CRP, the red dots with blue line indicates the TK1a assay and blue dots with black line indicates the combination of TK1a and CRP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To determine the combined effect of TK1a or TK1p with CRP, a combinational ROC analysis was performed. This ROC analysis showed that the combination of TK1-ELISA and CRP (NI/TK1p) had an AUC of 0.97 with a sensitivity of 0.88 and a specificity of 0.95 (Fig. 3A), and that the combination of TK1a and CRP (NI/TKa) had an AUC of 0.98 with a sensitivity of 0.85 and a specificity of 0.95 (Fig. 3B). The AUC for NI/TK1p and NI/TK1a ($P = 0.009$) was significantly higher than for TK1p and TK1a alone ($P = 0.005$). There was no significant difference between NI/TK1p with TK1-ELISA and TK1/TK1a with TK1a ($P = 0.99$). The results presented here indicate that the NI/TK1p combination is as effective as the NI/TK1a combination for distinguishing dogs with hematological malignancies from healthy dogs.

3.5. Survival analysis

Survival analysis using Kaplan-Mayer curves for dogs with hematological malignancies showed significant differences in survival time (ST) for groups with different initial TK1a, TK1p, and CRP values. Dogs with high pretreatment TK1p levels (>1.5 ng/mL) had a shorter ST than did dogs with low TK1p levels (<1.5 ng/mL; $p = 0.0075$, Fig. 4A).

Dogs with TK1a levels above 3 pmol min/mL had a shorter ST than did dogs with TK1a levels below 3 ($p = 0.01$, Fig. 4B). The CRP values over 20 mg/L were associated with shorter STs ($p = 0.006$, Fig. 4C). In the analysis of TK1a, TK1p, and CRP, one outlier was censored due to the small dataset (1/49). The most important survival factor was whether the dog had received any treatment, with the prognosis being better if the dog received treatment with multiple agents. Dogs that received no or only single-agent treatment had shorter STs ($p < 0.0001$). Treatment was highly significant even if dogs euthanized close to diagnosis were excluded from the analysis ($p < 0.0001$). The median STs for the different treatment options were no treatment, 12.5 days; prednisolone, 73 days; doxorubicin alone, 167 days; L-asparaginase + prednisolone, 92.5 days; doxorubicin-based protocol, 224 days; doxorubicin-based protocol + lomustine, 402 days. There were also other factors that affected prognosis. Patients with high age (over 8 years) lived shorter than patients below the age of 8 years ($P = 0,01$). CRP values over 25 mg/L were associated with shorter survival times ($p = 0,003$) . In the analysis for TK1a, TK1p and CRP an outlier (1/49) was excluded. Dogs with suspected malignant cells in peripheral blood at first visit had shorter survival times (p = 0,0004). Dogs that were of a median or large breed lived shorter than a dog of a small breed (small bread 0-15 kg, median 15-25 kg, large bread 35-kg) (p = 0,006). Survival times were shorter for dogs with b-symptoms at first visit (p = 0,004). If the tumor was considered high or low grade, or the dogs gender did not affect

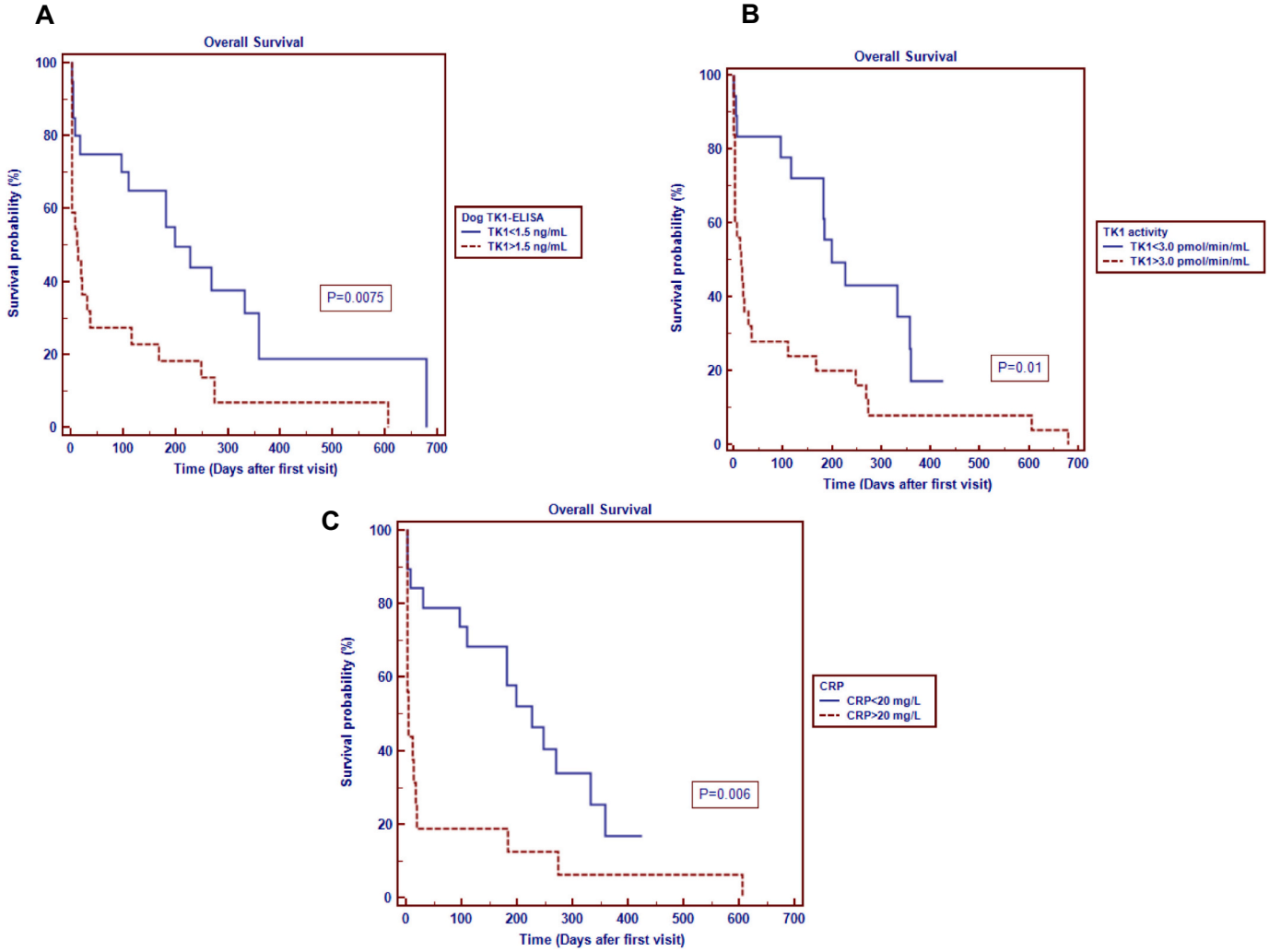

Fig. 4. Kaplan-Meier survival curves of dogs with hematological malignancies using (A) TK1-ELISA assay values for dogs with TK1p levels <1.5 ng/mL (blue line, mean survival time [ST] 261 days) versus dogs with TK1p levels >1.5 ng/mL (red line, mean ST 91 days; $p = 0.0075$). (B) TK1a assay values for dogs with TK1a levels <3.0 pmol/min/mL (blue line, mean ST 233 days) versus dogs with TK1a levels >3.0 pmol/min/mL (mean ST 100 days; $p = 0.01$). (C) CRP levels <20 mg/L (blue line, mean ST 219 days) and CRP levels >20 mg/L (red line, mean ST 71 days; $p = 0.006$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

survival times.

3.6. Case report describing the monitoring of dogs undergoing chemotherapy using TK1 activity (TK1a) and TK1 protein (TK1p) determinations

Twenty dogs that underwent treatment were used to establish the correlation of TK1a and/or TK1p with clinical outcome during treatment. Five dogs with the most complete datasets of TK1 and CRP depending on response to treatment were chosen to illustrate the principle. Four dogs with malignant lymphoma and one dog with acute leukemia were treated with a doxorubicin-based multi-agent protocol, with some also receiving lomustine after relapse. Serum samples were taken from the same individuals during chemotherapy. Fig. 5 presents the data concerning these dogs.

The results can be summarized as follows: TK1a, TK1p, and CRP values were lower when the dogs were in complete remission (CR) and they increased when they relapsed. The dogs were considered in CR when all palpable lymph nodes were normal in size. Median TK1p, TK1a, and CRP levels were significantly higher before treatment than in dogs in remission, their fluctuations being correlated with the clinical disease outcome.

4. Discussion

The present results establish that using TK1a and TK1p as biomarkers in dogs with hematological malignancies has significant diagnostic capacity. Overall, in dogs with hematological malignancies, about 74% of the TK1a and 68% of the TK1p determinations were above the cut-off values versus in the healthy dogs.

Lymphomas can be classified as high-, intermediate-, and low-grade disease (Valli et al., 2013). Patients with low-grade disease exhibit relatively long ST. Patients with low- and intermediate-grade lymphomas have significantly lower TK1p and TK1a levels and longer ST, whereas patients with high-grade lymphomas have significantly higher TK1p and TK1a levels and shorter ST. Of all dogs with malignant lymphomas, about 60% have TK1p and TK1a levels above the cut-off values.

TK1p and TK1a levels are clearly correlated to disease status. This is probably among the most important uses of TK1 in clinical settings. When frequently monitored, a TK1 level rise can predict disease progress before overt clinical signs are present (Boye et al., 2019; Selting et al., 2015; von Euler et al., 2004). Recently, Boye et al. (2019) showed that the percentage increase from an earlier TK1 measurement accurately predicted relapse at least one month before clinical signs were overt. Although the percentage increase is not calculated here, examples of the typical patterns of TK1a, TK1p, and CRP in a clinical setting with treated lymphoma/leukemia are shown in Fig. 5, clearly supporting Boye et al.'s (2019) findings. In this setting, even dogs with relatively low initial TK1 levels (e.g. as in many T-cell lymphomas) would benefit from serial TK1 measurements, as the change from the individual baseline predicts the clinical behavior. Although it remains to be proven in larger clinical studies in dogs with lymphoma, there is likely an animal welfare advantage and survival benefit if rescue treatment is given to dogs with a lower tumor burden. Serum tumor biomarkers (e.g. TK1) offer a non-invasive way to examine the animal, compared with advanced diagnostic imaging and multiple cytology testing in apparently normal-sized lymph nodes, liver, and spleen. This allows collection of information on the amount of tumor material before overt clinical signs of lymphoma

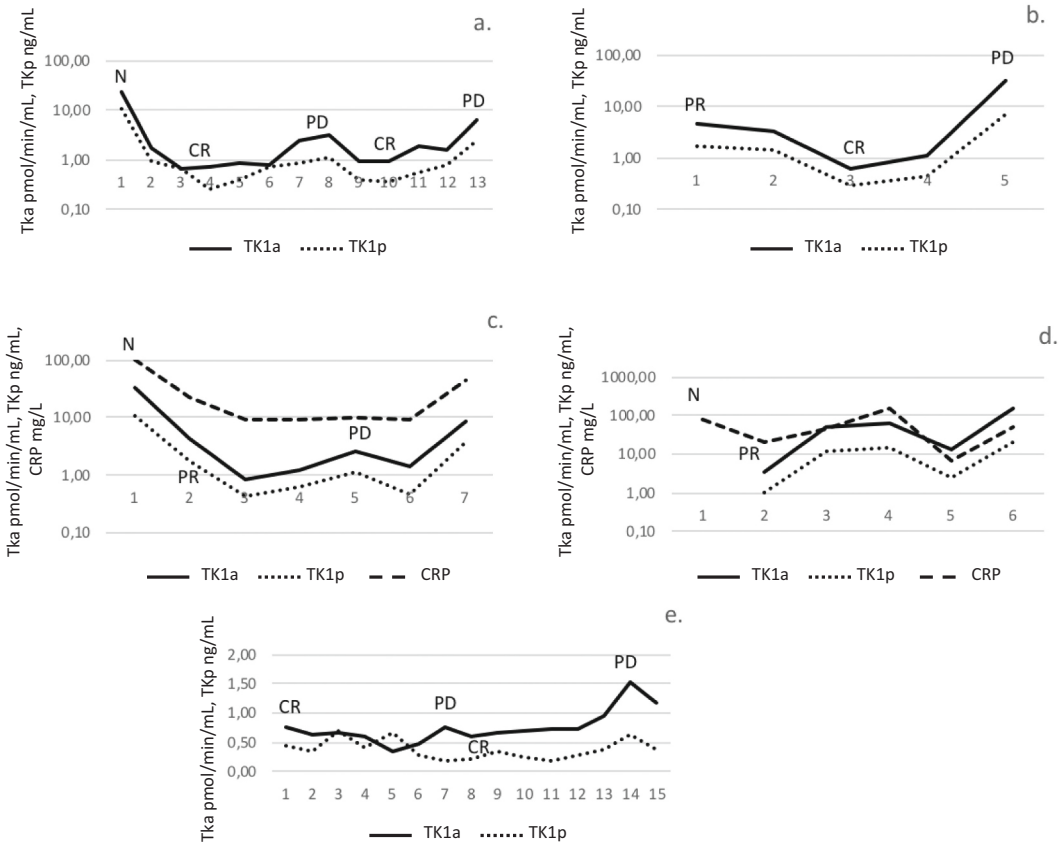

Fig. 5. Monitoring using TK1a, TK1p, and CRP levels of dogs undergoing chemotherapy. The two panels show typical patterns in the clinical situations, with the TK1a and TK1p levels particularly reflecting clinical response. Note that the *y*-axis has a log scale except for in graph e. TK1a is measured in pmol/min/mL, TK1p in ng/mL, and CRP in mg/L. The *x*-axis shows the sampling points during treatment. N = naïve disease, CR = complete remission, PR = partial remission, and PD = progressive disease. More detailed analysis of this dataset is presented in Supplemental Table 1, which describes possible decision points for individual patients.

are presented to the clinician and/or dog owner without using diagnostic procedures that are difficult, more costly, and increase the risk of sampling morbidities.

In this study, some lymphomas were found to have low initial TK1 concentrations. These patients have indolent and low-grade disease, and some have different anatomical manifestations, such as cutaneous lymphomas and lymphomas affecting the gastrointestinal tract. However, a limitation of this study is that most of the lymphomas were not immunophenotyped. Typing the lymphomas would have helped in further categorizing the lymphomas that have low TK1a, TK1p, as well as low CRP levels. Probably most of them were T-cell lymphomas, since it has been observed in earlier studies that T-cell lymphomas generally have lower TK1 levels (Boye et al., 2019; Selting et al., 2016).

Two out of three large granular cell lymphomas in this study had clearly elevated TK1a and TK1p values even though they were of T-cell origin. Canine lymphomas are a complex and heterogeneous group consisting of many different types; not all of them are easy to diagnose and monitor since the TK values are under or very close to the normal range. Some of the dogs in this study were not fully clinically staged. More thorough staging could have shown whether there is a correlation between clinical stage and TK1a, TK1p, and CRP levels, as demonstrated in previous studies (Boye et al., 2019; Selting et al., 2016). The type of treatment as well as TK1a, TK1p, and CRP levels were clearly correlated to ST, and dogs with very high TK values usually have a poor prognosis regardless of treatment. The correlation between the presence of B symptoms and poor prognosis has been demonstrated earlier, so the association found here was expected (figure not shown, data on B symptoms in Table 2).

This study shows that CRP level can be a valuable complement to TK1a and TK1p levels in monitoring dogs with hematologic malignancies during treatment and can also help in prognostication. Inflammation and cancer are closely linked, and dogs with rapidly growing high-grade tumors probably have a greater inflammatory component and hence higher CRP than dogs with slower-growing low-grade tumors. in agreement with earlier studies(Selting et al., 2016; Selting et al., 2015).

Combination analysis of TK1p or TK1a with CRP showed higher sensitivity than either TK1p or TK1a alone, suggesting that a panel of biomarkers including TK1 and CRP may aid in the management of various canine malignant diseases and thus serve as a valuable tool in veterinary medicine. Most previous studies were based on TK1a assays that need radioactive material or complex assay methods. However, the TK1-ELISA overcomes these limitations, which is important in developing antibody-based routine clinical chemistry assays for TK1. Used alone CRP is not a reliable marker enough to address prognosis nor to diagnose hematological tumors in dogs. In this article, we have shown that adding CRP-levels to the cell proliferation marker TK1 indicates that the utility of a combination improves the clinical value. To validate this finding further, larger studies need to be undertaken.

5. Conclusions

Thymidine kinase 1 (TK1), an enzyme involved in DNA precursor synthesis, is used as a serum biomarker in cancer diagnostics in both human and veterinary medicine. The present results establish that using TK1a and TK1p as biomarkers in dogs with hematological malignancies has significant diagnostic capacity. Overall, in dogs with hematological malignancies, about 74% of the TK1a and 68% of the TK1p determinations were above the cut-off values versus in the healthy dogs. Examples of the typical patterns of TK1a, TK1p, and CRP in a clinical setting with treated lymphoma/leukemia are presented and clearly support suggestion that percentage increase can guide clinical decision making. In this setting, even dogs with relatively low initial TK1 levels (e.g. as in many T-cell lymphomas) would benefit from serial TK1 measurements, as the change from the individual baseline predicts the clinical behavior. Although it remains to be proven in larger clinical studies in dogs with lymphoma, there is likely an animal welfare advantage and survival benefit if rescue treatment is given to dogs with a lower tumor burden.

Declaration of Competing Interest

Staffan Eriksson is a co-inventor of a TK1 activity patent licensed to DiaSorin Inc. and several patents owned by AroCell AB.) He is a co-founder, shareholder, and consultant to the company AroCell AB, Uppsala. There is a U.S. patent pending for the antibodies and assay procedures described in this manuscript: # 16323–244: Determination of Non-Human Mammal TK1 protein levels, with K.K. Jagarlamudi, H. Rönnberg, and S. Eriksson as inventors. Henrik Rönnberg is member of the scientific advisory board and shareholder of Alertix. This does not alter the authors' adherence to all the Veterinary Journal's policies on sharing data and materials.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2022.02.019.

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