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Serum thymidine kinase 1 and C-reactive protein as biomarkers for screening clinically healthy dogs for occult disease

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Abstract

Thymidine kinase (TK1) is a biomarker that correlates well with diagnosis and prognosis in certain canine cancers. Canine C-reactive protein (cCRP) is a widely accepted marker of inflammation correlated with increased risk and severity of various diseases. We evaluated serum TK1 and cCRP concentrations in apparently healthy dogs ($n = 360$). All dogs were followed up for a minimum of 6 months by health questionnaire. All dogs with cancer were identified using a proprietary dual-biomarker algorithm [termed Neoplasia Index (NI)]. Specificity of positive NI is 0.91 and high positive is 0.98. All-cause mortality was 20% in dogs with elevated cCRP and 3% in dogs with low cCRP. The performance of serum TK1 and cCRP as tools for screening for occult cancer is improved when evaluated together. Serum TK1 and cCRP (unified in the NI) are useful in the screening of occult canine cancer. cCRP is useful in screening for other serious diseases.

Keywords
biomarker, cancer, cCRP, dog, inflammation, neoplasia, Neoplasia Index, screening, TK

Introduction

There is great demand for accurate and minimally invasive health screening methods in veterinary medicine to detect occult disease. Detection of underlying pathology prior to the development of outward signs of disease can improve efficacy of treatment in many diseases, and early detection of neoplasia improves treatment success for most cancers. Thymidine kinase 1 (TK1) and canine C-reactive protein (cCRP) are promising biomarkers for detection of occult disease. Serum TK1 is an effective diagnostic aid for cancer in dogs,[1–5] and high serum concentration of cCRP is well documented in dogs with cancer and various other diseases associated with systemic inflammation.[6–14]

TK1 is a cytosolic enzyme involved in the salvage pathway for synthesis of thymidine, a DNA precursor. TK1 is most notably associated with the DNA synthesis phase of the cell cycle (S phase) and its expression is limited to proliferating cells. Normal concentrations in dogs are 0–6 U L^{-1} (consistent with both previously published studies and our internal data).[2] Accordingly, it is increasingly expressed in malignant cells which are characterized by rapid cell replication. Serum TK1 has been shown in several studies to be increased in a variety of cancers, both benign and malignant.[6,15,16] Historically, research has been centred on haematopoietic cancers such as leukaemia and lymphoma where serum TK1 concentration can be markedly increased, and correlates with stage of disease and prognosis.[1–4] More recently, high serum TK1 was documented in canine hemangiosarcoma.[6] However, solid tumours including other sarcomas do not have consistently increased TK1 concentrations, limiting its utility as a sole biomarker for detecting all cancer histologies. Previous data found TK1 range and mean value for selected solid tumours: transitional cell carcinoma

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(0–73.7, mean 7.37 U L^{-1}) and osteosarcoma (0.3–16.1, mean 2.7 U L^{-1}) (VCS 2007, unpublished data). Despite multiple studies showing increased TK correlating with stage and prognosis in dogs with lymphoma, a recent study found more than half of 73 dogs evaluated had normal TK at presentation, and that TK did not correlate with stage. However, limitations with that trial included inconsistent sample handling with some serum stored on gel for up to 24 h before freezing and later thaw and processing, a lack of control group to confirm performance of the radioimmunoassay and relatively few stage 3 or lower cases with the vast majority of dogs having stage 4 or 5 lymphoma, and some dogs lacking complete staging.[17]

TK1 can also be increased in pre-malignant conditions, as well as viral infections. Because viruses have a smaller genome, some (such as cytomegalovirus) can induce transcription of host TK1 to ensure their survival. Other viruses (such as herpes viruses) have their own, genetically unique, TK enzyme.[18] Viral diseases are less commonly recognized in dogs as compared with humans. Because TK1 can be released from rapidly dividing cells, or from those that die during replication, inflammatory conditions could theoretically cause minor increases in TK1. Our group has also noted a small number of dogs with minor increases in TK1 and concurrent positive testing for rickettsial disease (unpublished data).

cCRP is a well-accepted biomarker of inflammation produced predominantly in the liver in response to pro-inflammatory cytokines as part of the acute-phase response. Serum cCRP is the most thoroughly investigated acute-phase protein in dogs, and is an effective measure of systemic inflammation; cCRP correlates to both the severity and duration of the inflammatory stimuli. The limitation of cCRP as a biomarker is that it is not disease specific, with high serum concentrations found in a variety of diseases associated with systemic inflammation.[12] In human medicine, CRP is often used in panels of biomarkers, and can identify a subtle increase in risk of developing colorectal cancer which is a model of multistage carcinogenesis.[19]

Evaluation of cCRP in combination with TK1 is rational given the relationship between cancer and inflammation. The inflammatory response orchestrates host defences to infection, trauma, toxins or other tissue damaging events and mediates tissue repair and regeneration. Chronic inflammation promotes the development of dysplasia and ultimately predisposes to the development of cancer. Additionally, inflammation plays an essential role at each stage of cancer development,[20,21] and the success of tumour development correlates directly with the degree of the associated inflammation. Proposed mechanisms include release of growth factors and cytokines that promote angiogenesis and tissue invasion/matrix degradation, as well as local suppression of anticancer effector cells. [22,23]

The use of cancer biomarkers is common in human medicine. TK1 has also been investigated for its value in screening clinically healthy people for occult disease. A recent study from China reported the association of TK1 with health and occult disease in 35 365 people who presented for wellness checks and considered themselves healthy. TK1 concentrations were higher in rural oil field workers, than in urban dwellers and this corresponded with higher incidence of pre-malignant conditions, refractory anaemia, fatty liver disease and obesity. Additionally, city dwellers with high TK1 had higher incidence of malignant, pre-malignant and hyperplastic conditions (especially of gastric, liver and breast or prostate origin) than those with normal TK1. Cases of occult malignancy in city dwellers were uncommon with four cases confirmed (out of 198 people with high TK1). No malignancies were detected in city dwellers with normal TK1.[23] TK1 has also been useful in preliminary investigations screening people for breast and nasopharyngeal cancer.[24,25]

The objective of this study was to evaluate serum TK1 and cCRP in a group of apparently healthy dogs with no history of cancer, and follow-up these dogs with health questionnaire for a minimum of 6 months and up to 1 year, so as to assess the diagnostic utility of these biomarkers to detect occult disease. Our hypothesis was that by combining cCRP with TK1, low-end sensitivity of TK1 would improve without resulting in loss of specificity for the diagnosis of cancer, and that cCRP alone would be valuable in detecting other serious

diseases associated with inflammation in dogs prior to the development of overt clinical signs of disease.

Materials and methods

Animals

Owners of dogs within German shepherd and Golden retriever breed clubs were recruited. Dogs with no visible signs of cancer or history of cancer or other serious disease were eligible for inclusion in this screening study. Dogs with a familial history of cancer were not excluded. The study was designed to sample dogs aged 5 years and older such that the control population would be comparable with the diseased population. Organized blood collection at breed club meetings and dog shows in Missouri, Minnesota, California, Massachusetts and Florida were conducted ($n = 140$). In addition, by communicating through breed clubs, owners across the USA were encouraged to allow veterinarians to submit a blood sample if the dog was not present at a show or group blood draws ($n = 220$). Each dog owner completed a questionnaire about diet and health history including lack of non-specific or specific clinical signs of any disease at the time of blood collection. Dogs were not examined by a veterinarian at time of enrolment and health status was established using results of health questionnaires at baseline, 4, 6 and 12 months after enrolment for the purpose of these comparisons. Those samples drawn at veterinary offices were not necessarily in the context of a physical examination. The study was approved by the Clinical Studies Review Committee (Tufts). Institutional Animal Care and Use Committee approval was not required by the University of Missouri at the time of this study. Additionally each owner was required to provide signed consent.

Sample collection

Blood was collected via venipuncture by a veterinarian or veterinary technician and centrifuged within 1 h of sample collection, and a minimum of 0.5 mL serum was harvested. The serum was placed in an airtight, freezer-resistant plastic tube and stored at $-20\,^{\circ}C$ or colder. Tubes were coded so that the identity of the sample was known only to the investigator; the laboratory was blinded.

Monitoring

Dogs were followed up, by direct contact with pet owner, for a minimum of 6 and maximum of 12 months for signs of cancer or other disease. Health status was recorded at 4, 6 and 12 months after initial blood collection. Dogs whose initial serum TK1 was greater than $30.0\,U\,L^{-1}$ were referred for diagnostic imaging (thoracic radiography and abdominal ultrasound, subsidized by the trial) to determine if neoplasia was present. Dogs whose initial TK1 was greater than 6.0 but less than $30\,U\,L^{-1}$ were re-sampled 60 days after baseline blood collection, and if values increased or remained greater than $6.0\,U\,L^{-1}$, they were referred for the same imaging. Medical history was obtained from the primary veterinarian for all dogs that died during the study or developed cancer or other disease. When possible, an additional serum sample was obtained at the time of cancer diagnosis for measurement of TK1 and cCRP for comparison to values obtained upon initial blood collection.

TK1 assay

Serum TK1 was evaluated by a commercial laboratory (Veterinary Diagnostics Institute, Simi Valley, CA, USA). The LIASON TK assay (DiaSorin, Stillwater, MN, USA) is an indirect, modified two-step, competitive chemiluminesence immunoassay (CLIA) for the quantitative determination of TK1 in serum and has been previously validated for use in dogs.[4] The upper limit of normal is $6\,U\,L^{-1}$ (the 90th percentile is $5.7\,U\,L^{-1}$, median $1.9\,U\,L^{-1}$, for our previous data and other publications use this limit).[2,4,5]

cCRP assay

Serum cCRP was evaluated by a commercial laboratory (Veterinary Diagnostics Institute). The TECO® Canine cCRP assay (TECOmedical group, Sissach, Switzerland) is a canine-specific sandwich enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of cCRP in canine serum and has been previously validated in this laboratory and

by others[26] for use in dogs. The assay has low intra- and inter-assay precision of 4.3 and 6.0%, respectively, and correlates well with another commercially available cCRP assay ($r = 0.976$). The upper limit of normal is 7 mg L^{-1} (the 90th percentile is 6.7 mg L^{-1}, median 1.9 mg L^{-1}, for our previous data and other publications use this limit.[13])

Disease classifications

Dogs with malignant neoplasia were classified in the 'cancer' group. Dogs with benign neoplasms or no evidence of neoplasia during the follow-up period were classified in the 'non-cancer' group. Neoplasia was confirmed by cytology or histopathology in seven cases. In an additional four cases, this was not possible and medical records from the primary veterinarian were obtained to categorize the neoplasia by laboratory blood work, diagnostic imaging and clinical findings. One dog had marked hepatosplenomegaly with ultrasound characteristics consistent with lymphoma and was responsive to prednisone, a second dog had hypercalcaemia and marked increase in parathyroid hormone (PTH), the third dog died acutely from hemoabdomen with a bleeding splenic mass and the fourth also had acute abdominal distention with anaemia, pallor and shock. In addition, statistics were repeated after censoring these four cases and this did not change the results so they were retained for the results reported here. For reporting of mortality, dogs that succumbed to non-neoplastic disease were classified in a group referred to as the 'Other Serious Diseases Group'. For the purpose of this study, a serious disease was defined as a dog whose death or euthanasia was from a cause other than cancer.

Algorithm development

After the initial blood collection from large breed clubs, a subgroup of this study ($n = 254$) was used as normal dogs along with a separate diseased cohort to develop a diagnostic algorithm. The diseased cohort included previously reported dogs with malignant ($n = 42$) or benign ($n = 20$) neoplasia.[6] TK1 and cCRP values were grouped into ranges that optimized separation between normal, benign and cancer and then categorized by unitless discrete values (0, 1, 2, etc.) to prevent high values of one biomarker overly influencing the value of another. As it is known that cCRP is sensitive but not specific to cancer and that TK1 is specific but not highly sensitive to localized solid cancers, particular focus was given to improve the low-end sensitivity of TK1 without losing specificity. Logistic regression was performed on the discretized data with resulting weighting coefficients for each biomarker. A Neoplasia Index (NI) was created which is the sum of each discretized biomarker multiplied by its coefficient. As a result, NI can range from 0 to 9.

Statistical analysis

Statistical analysis was performed using commercially available software (MedCalc Software, version 12.3, Belgium). A Kruskal–Wallis one-way analysis of variance was used to compare serum TK1, cCRP and NI results among cancer and non-cancer dogs. A Mann–Whitney rank sum test was used to compare age and sex between cancer and non-cancer groups. A receiver-operating characteristic (ROC) curve was used to determine area under the curve (AUC) and select the optimum cut-off value that maximized the Youden's J statistic (sensitivity + specificity − 1) for sensitivity and specificity reporting. Likelihood ratios were used for interval performance. For the purposes of sensitivity and specificity reporting, dogs with cancer and a positive test result were considered true-positives. Conversely, dogs without cancer and a negative test result were considered true-negatives. Likelihood ratios are defined as the probability of a positive test result in those with cancer divided by the probability of that same result in those without cancer. A *P*-value of 0.05 was considered significant for all analyses.

Results

Study population

A total of 378 dogs were sampled; 18 were disqualified due to inadequate sample volume ($n = 12$), known cancer at the time of blood collection ($n = 4$, owners had erroneously requested samples to be submitted), or lost to follow-up ($n = 2$).

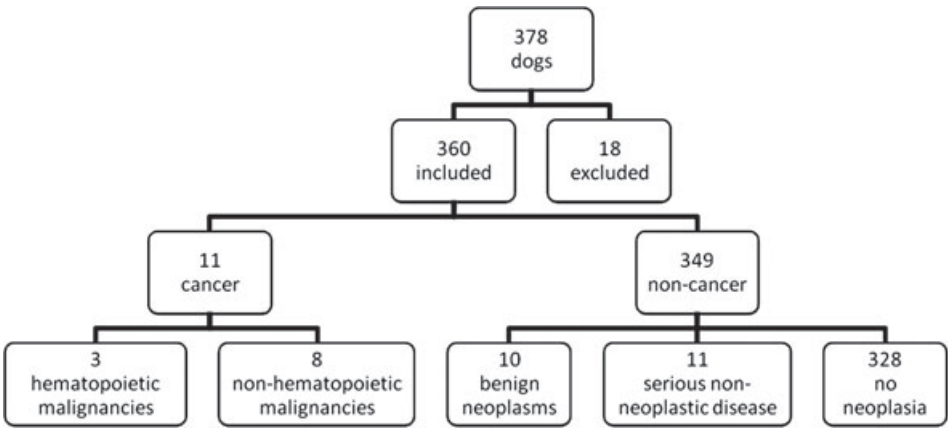

Figure 1. Flowchart depicting number of dogs in each category of cancer and non-cancer cohorts.

A total of 360 dogs met the criteria and were subsequently enroled. Median follow-up time was 198 days (dictated largely by the study design, most dogs had follow-up to at least 6 months) with a range of 18–416 days. All dogs with follow-up less than 6 months died due to disease.

Over the course of the study, 11 dogs developed malignant cancer ('Cancer' group) and 10 had benign neoplasms. The remaining 339 were not diagnosed with neoplasia during the follow-up period and together with the benign neoplasms and non-neoplastic illness constitute the 'non-cancer' group (Fig. 1). Of the 11 malignancies, 3 were haematopoietic cancers and 8 were non-haematopoietic cancers (Table 1). One cancer (parathyroid tumour) was diagnosed because the sample was concurrently analysed for calcium, vitamin D and parathyroid concentrations as part of an unrelated study, and parathyroid hormone and calcium concentrations were very high. The overall incidence of cancer was 3%. Signalment information is listed in Table 2.

There was no significant difference between the cancer and non-cancer dogs in terms of breed and sex, however, dogs with cancer were older than the non-cancer dogs with median age of 9.8 and 7.0 years, respectively ($P = 0.004$). There was no significant difference between the malignant cancer and benign neoplasm, dogs in terms of breed and sex. The cancer group was older than the dogs with benign neoplasms, with a median age of 9.8 and 6.1 years, respectively, although this difference did not reach significance ($P = 0.08$).

Table 1. Types of cancer diagnosed in the dogs in the cancer group

Histopathologically/cytologically confirmed cancers		
Total		7
Types	Leukaemia	
	Hemangiosarcoma ($n = 2$)	
	Abdominal sarcoma	
	Lymphoma	
	Intestinal sarcoma	
	Anal sac adenocarcinoma	
Clinically apparent		
Total		4
Types	Hemangiosarcoma ($n = 2$)	
	Lymphoma	
	Parathyroid Tumour	
Total cancers		11

Comparison of TK1 and NI among cancer

Serum TK1 concentration was significantly greater in the cancer group (median, Q1, Q3: 9.2, 3.8, 20.0 U L^{-1}, respectively) compared with the non-cancer group (2.1, 1.1, 4.0 U L^{-1}, respectively, $P < 0.001$). Serum cCRP concentration was significantly greater in the cancer group (median, Q1, Q3: 9.7, 3.6, 18.4 mg L^{-1}, respectively) compared with the non-cancer group (2.0, 1.2, 3.6 mg L^{-1}, respectively, $P < 0.001$). With NI, values were significantly higher in the cancer group (median, Q1, Q3: 6.9, 5.8, 9, respectively) compared with the non-cancer group (2.1, 0, 1, respectively, $P < 0.001$; Fig. 2).

On the basis of evaluation of the ROC curve, the AUC for TK1 in differentiating dogs with cancer at 6 months prior to the onset of signs was 0.84 [95%

Table 2. Comparison of age, sex, breed and outcome in cancer and non-cancer dogs

	Non-cancer group		Cancer group
Signalment information	**Normal**	**Benign**	**Malignant**
N	339	10	11
Age median (range)	7 (0.4–14.5)	6 (5–11)	10 (5–15.4)
Sex	F (73), FS (104), M (92), MN (69)	F (5), FS (4), M (1), MN (0)	F (3), FS (4), M (1), MN (3)
Breed	German Shepherd dog (152)		
	Golden Retriever dog (175)		
	White Shepherd dog (8)	German Shepherd dog (6)	German Shepherd dog (5)
	Portuguese Water dog (4)	Golden Retriever dog (4)	Golden Retriever dog (6)
Died	18	1	8

F, female; FS, female spayed; M male; MN, male neutered.

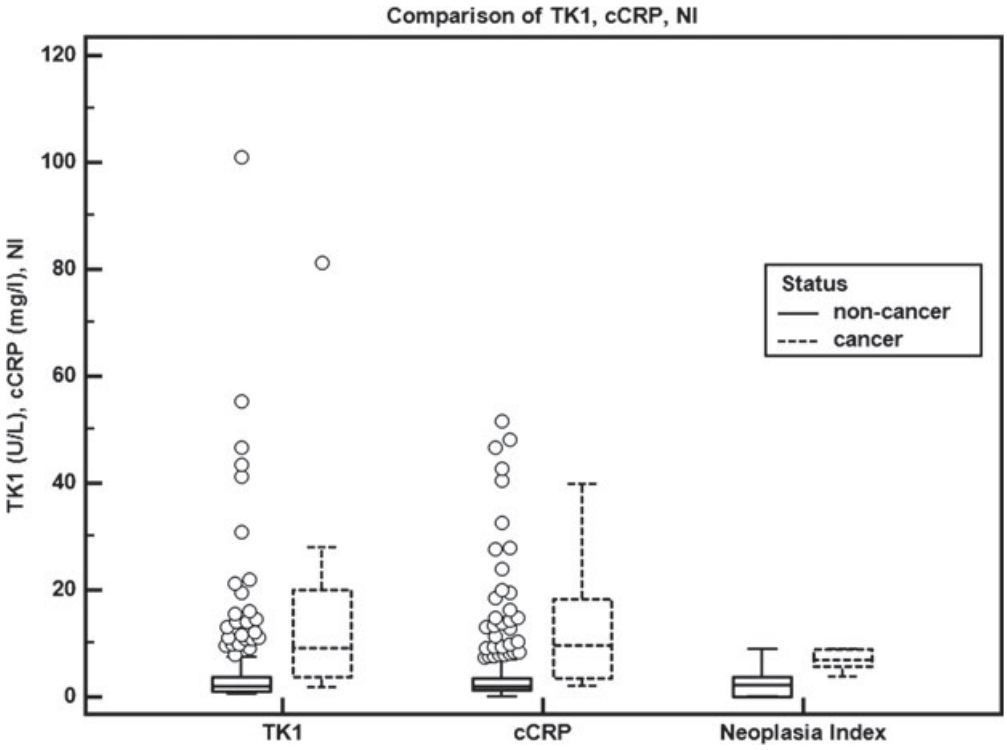

Figure 2. Box and whisker plots comparing serum concentrations of TK1, cCRP and NI between cancer ($n = 11$) and non-cancer dogs ($n = 349$). The upper and lower edges of the box represent the 75th and 25th percentiles, respectively, whereas the line within the box is the median value. Whiskers represent the largest and smallest values. Outliers are represented by open circles.

confidence interval (CI), 0.80–0.88). Using NI, the ROC AUC in differentiating dogs with cancer at 6 months was 0.93 (95% CI, 0.90–0.96; Fig. 3). This difference in ROC AUC between TK1 and NI was significant ($P = 0.038$). For TK1, the sensitivity was 0.73 (95% CI, 39.0–94.0%) and specificity was 0.84 (95% CI, 80.0–87.9%) at a cut-off of $\geq 4.9\ \mathrm{U\,L^{-1}}$. For NI, the sensitivity was 0.82 (95% CI, 48.2–97.7%) and specificity was 0.91 (95% CI, 83.0–93.4%) at a cut-off of ≥ 5.8.

Interval performance is listed in Tables 3 and 4. For TK1, there were three false-negatives which consisted of two hemangiosarcomas and one intestinal sarcoma. For NI, there were two false-negatives which consisted of one hemangiosarcoma and one intestinal sarcoma. Overall mortality rate with positive TK1 consisted of 11 dogs (20%) of

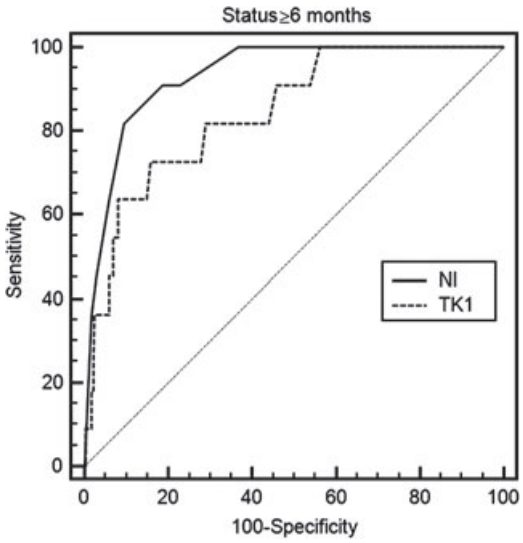

Figure 3. ROC curves comparing the diagnostic sensitivity and 100-specificity of TK1 (dotted line) and the NI (black line) for differentiating dogs with cancer diagnosed within 6 months of measurement. The grey line represents an AUC of 0.5. NI had a significantly higher AUC (0.93) than TK1 (AUC 0.84, $P = 0.038$).

which 6 died or were euthanized of cancer. Overall mortality with positive NI consisted of 11 patients (33%) of which 7 died or were euthanized of cancer.

Blood specimens were obtained from four dogs at the time of cancer diagnosis to observe the change in TK1 and cCRP that occurs from initial sampling when the dog showed no overt signs of disease and at the time of diagnosis 3–5 months later (Fig. 4). In all the four cases, TK, cCRP and NI increased.

Benign versus malignant neoplasia

Dogs with benign neoplasia ($n = 10$), a subset of the non-cancer dogs, included trichoepithelioma, hyperplasia of lymph nodes, fibrous lipoma, fibroma, haemangioma, benign mixed mammary ($n = 3$), benign cutaneous mass and inflammation/keratin debris. On the basis of evaluation of an ROC curve, the AUC for TK1 in differentiating dogs with malignant cancer from those that had benign neoplasms was 0.66 (95% CI, 0.42–0.85). Using NI, the ROC AUC in differentiating dogs with malignant cancer from those that had benign neoplasms was 0.85 (95% CI, 0.62–0.97, Fig. 5). This difference in ROC AUC between TK1 and NI was significant ($P = 0.019$). For TK1, the sensitivity was 0.70 (95% CI, 34.8–93.3%) and specificity was 0.73 (95% CI, 34.0–94.0%) at a cut-off of $<4.5\ \mathrm{U\ L^{-1}}$. For NI, the sensitivity was 0.80 (95% CI, 44.4–97.5%) and specificity was 0.91 (95% CI, 58.7–99.8%) at a cut-off of <5.8.

All-cause mortality

Over the study period 27 dogs died or were euthanized; 8 from cancer and 19 from other causes (Table 5). Serum cCRP concentrations were significantly greater in the all-cause mortality

Table 3. Interval performance for TK1 and Neoplasia Index in dogs with cancer and no cancer

Interval	Cancer	Non-cancer	Likelihood ratio	All-cause mortality	Cancer mortality	Sensitivity	Specificity
TK1							
0–1.7	0	153	0.00	8	0	1.00	0.00
1.8–3.0	2	89	0.71	5	1	1.00	0.44
3.1–4.8	1	52	0.61	3	1	0.82	0.69
4.9–9.0[a]	2	30	2.12	5	2	0.73	0.84
9.1–19.4	3	17	5.60	3	2	0.55	0.92
≥19.5	3	8	11.90	3	2	0.27	0.98
Total	11	349		27	8		
Neoplasia Index							
0–2.0	0	153	0.00	8	0	1.00	0.00
2.1–4.1	1	116	0.27	5	1	1.00	0.63
4.2–5.7	1	47	0.68	3	0	0.91	0.81
5.8–6.8[a]	2	12	5.29	1	0	0.82	0.91
6.9–8.9	3	15	6.35	5	3	0.63	0.94
≥9.0	4	6	21.15	5	4	0.36	0.98
Total	11	349		27	8		

[a]Youden index J cut-off.

Table 4. Comparison of TK1 versus NI in cancers confirmed by histology/cytology regardless of time interval, at 4-month health status check of all cancers detected at that interval, and at ≥6-month health status check of all cancers detected

		Positive		High positive	
TK1	**ROC AUC**	**Sensitivity at ≥4.9 U L^{-1}**	**Specificity at ≥4.9 U L^{-1}**	**Sensitivity at ≥19.5 U L^{-1}**	**Specificity at ≥19.5 U L^{-1}**
Confirmed	0.815	0.71	0.92	na	na
4-month status	0.903	0.89	0.84	0.33	0.98
≥6-month status	0.844	0.73	0.84	0.27	0.98

		Positive		High positive	
Neoplasia index	**ROC AUC**	**Sensitivity at ≥5.8**	**Specificity at ≥5.8**	**Sensitivity at ≥9.0**	**Specificity at ≥9.0**
Confirmed	0.940	0.86	0.90	0.29	0.98
4-month status	0.967	1.00	0.91	0.44	0.98
≥6-month status	0.933	0.82	0.91	0.36	0.98

na, not able to calculate due to lack of any increased values above this concentration.

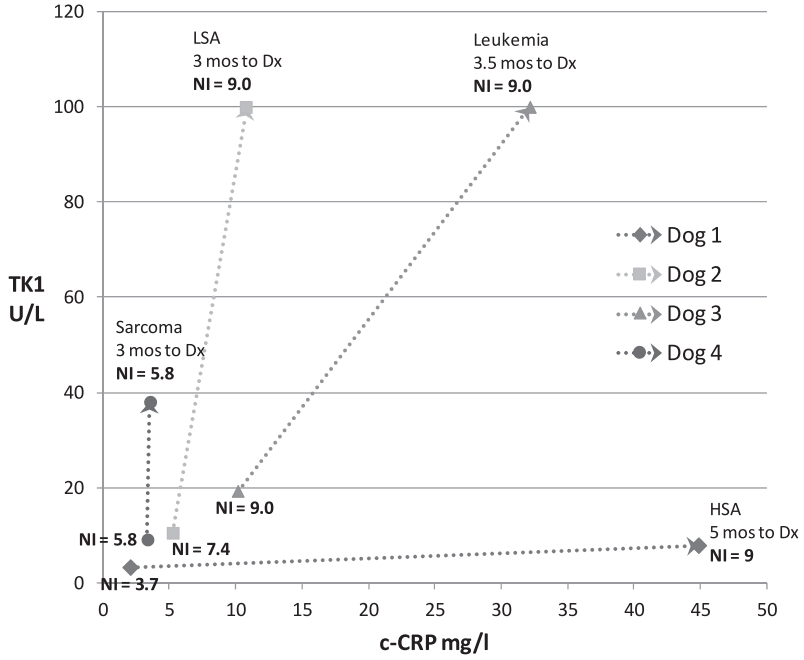

Figure 4. Comparison of baseline TK1 and cCRP in four dogs with cancer at first measurement and the time of cancer diagnosis.

group (median, Q1, Q3: 5.0, 2.4, 10.0 mg L^{-1}, respectively) to the alive group (1.9, 1.2, 3.3 mg L^{-1}, respectively, $P < 0.001$). There was no correlation between age and cCRP concentration ($r = 0.08$). Using a cut-off of ≥3.8 mg L^{-1}, cCRP was high in 24% of the study population. Within the high cCRP group, the mortality rate was 20% during the study period compared with those that had cCRP ≤3.7 mg L^{-1}, the mortality rate was 3%. This resulted in an odds ratio of 6.3 ($P < 0.001$).

Discussion

Cancer is a leading cause of death in dogs. In the population reported here, designed to be geriatric and of high-risk breeds, the overall incidence of malignant cancer was 3%. Thus, a screening test must be both sensitive and highly specific. In this investigation we found that by combining TK1 and cCRP in an algorithm, the resultant NI was able to detect a variety of malignant cancer types

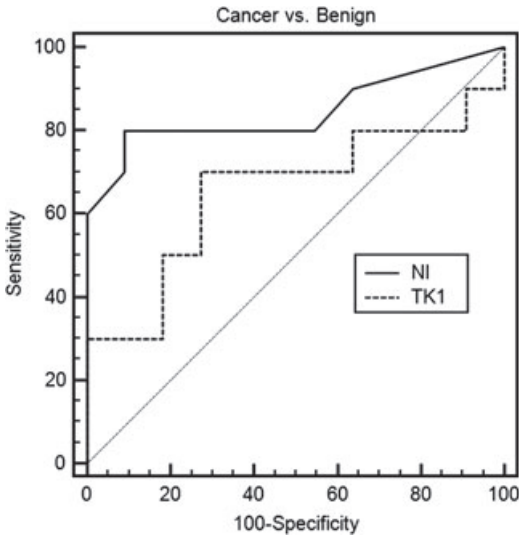

Figure 5. ROC curves comparing the diagnostic sensitivity and 100-specificity of TK1 (dotted line) and the NI (black line) for differentiating dogs with malignant cancer from those with benign neoplasms diagnosed within 6 months of measurement. The grey line represents an AUC of 0.5. NI had a significantly higher AUC (0.85) than TK1 (AUC 0.66, $P = 0.019$).

Table 5. Cause of non-survival in individual dogs listed from lowest to highest serum cCRP concentration at initial enrolment

Total deaths	Death due to cancer		Death due to other causes
27	8[a]		19
Causes of death	**cCRP (mg L^{-1})**	**TK1 (U L^{-1})**	**NI**
Degenerative myelopathy	0.7	**6.8**	4.2
Degenerative myelopathy	1.0	0.4	0.0
Old age	1.1	3.2	2.1
Leptospirosis	1.3	3.2	2.1
Unknown cause of death	1.6	3.0	2.1
Hemangiosarcoma[a]	2.0	3.4	3.7
Hepatitis	2.3	1.3	0.0
Old age	2.7	3.0	3.7
Unknown cause of death	2.7	**6.7**	**5.8**
Old age	**3.8**	1.4	0.0
Fibrous lipoma (benign)	**4.2**	**46.6**	**7.4**
Lame in back end	**4.2**	1.6	0.0
Perforated intestine	**4.5**	2.0	5.3
Old age	**5.0**	0.7	0.0
Lymphoma[a]	**5.2**	**10.6**	**7.4**
Stroke	**5.5**	1.6	0.0
Old age	**5.6**	0.4	0.0
Unknown cause of death	**6.5**	2.1	5.3
Lame in back end	**7.3**	0.7	0.0
Parathyroid cancer[a]	**9.7**	**4.9**	**6.9**
Leukaemia[a]	**10.1**	**19.4**	**9.0**
Hemangiosarcoma[a]	**10.2**	2.3	**6.9**
Seizures	**14.3**	**5.2**	**6.9**
Hemangiosarcoma[a]	**21.1**	**6.9**	**9.0**
Backend gave out	**23.9**	**19.4**	**9.0**
Lymphoma[a]	**29.3**	**28.0**	**9.0**
Hemangiosarcoma[a]	**39.8**	**81.2**	**9.0**

Bold = at/above cut-off.
[a]Death due to cancer.

with 82% sensitivity and 91% specificity 6 months prior to the onset of signs; at 4 months prior to the onset of signs, the sensitivity was found to be 100%. It is important to note that as all dogs did not undergo haematologic evaluation and diagnostic imaging, these data may overestimate specificity and sensitivity as false-negatives are possible and reclassification from a true- to a false-negative would affect both values. While it is possible that the increase in biomarkers was not a direct result of the underlying disease process, we were able to document rising biomarkers in dogs ultimately diagnosed with cancer within a short period of time. A very small number of dogs were detected which is expected in the study design, but required a large number of dogs to find these few cases, and will require additional studies to corroborate our findings. In our study the incidence of cancer was 3% which corresponds well with the recent study in people in which 2% of city dwellers with increased TK1 were diagnosed with an occult malignancy.[27] In addition, cancer is known to have a latent period during which neoplastic transformation has occurred but clinically evident disease has not yet been detected. Therefore it is likewise feasible that the increase in biomarker levels would preceed the clinical detection of disease. The results of this study are consistent with others, demonstrating that while TK1 is highly specific for certain malignant cancers, making it a useful rule-in diagnostic tool, sensitivity can be low in localized tumours such as sarcomas.[1,2,6] Low sensitivity (thus a high rate of false-negatives) is less than ideal when attempting to use TK1 as a screening biomarker prior to the onset of clinical signs of disease. Our study found that if the TK1 cut-off is lowered to improve sensitivity to 80% sensitivity at the 6-month mark (>3.3 U L^{-1}), the specificity quickly diminishes, with the false-positive rate increasing significantly to almost 30%. Low specificity makes differentiating benign from localized malignant

neoplasms particularly problematic (and to a lesser extent non-cancer from cancer), especially when serum TK1 concentrations are below 6 U L^{-1}. By using cCRP as a 'TK1 qualifier', especially when TK1 is less than 6 U L^{-1}, the algorithm developed in this study significantly reduces false-positives that otherwise would occur. When TK1 is qualified with cCRP, the false-positive rate drops to 9%. Using NI, all but two non-cancer were effectively separated from the cancer patients. Of the two false-positives one was diagnosed with fibrous lipoma and died shortly after diagnosis; unfortunately no postmortem examination was performed so occult neoplasia or other disease cannot be ruled out. The other false-positive was a dog with a history of pyometra that had a high TK1 3 months later and was diagnosed with lymph node hyperplasia on fine needle aspiration of enlarged abdominal lymph nodes. This dog has recently relapsed with high TK1 values and continues to be monitored. Thus the NI used in this study maximizes sensitivity and specificity, as well as interval likelihood ratios, for the screening of clinically healthy dogs for occult malignant cancer up to 6 months prior to the onset of clinical signs of disease, compared with the use of TK1 alone.

In addition to the utility of the dual biomaker approach of the NI as a diagnostic screening tool, NI was also shown to have greater prognostic value in this study compared with TK1 alone. The likelihood of both all-cause mortality and cancer increase as NI increases. For example, dogs with a NI above 6.9 had 100% cancer mortality as compared with 67% mortality when TK1 is above 9.1 U L^{-1}. This is logical as cancer proliferation (as measured by TK1) and systemic inflammation (as measured by cCRP) are high when NI is high. Our study also demonstrated the ability of cCRP, used alone, as a screening biomarker for other occult (non-neoplastic) diseases in this population of large breed dogs.

In this cohort of apparently healthy dogs, persistently high positive serum cCRP concentrations are most likely attributable to occult chronic inflammation, given the known association of CRP with inflammation, and the lack of acute illness dictated by the study design. Additionally, we found a strong association of high cCRP with all-cause mortality, suggesting that cCRP alone has prognostic utility in apparently healthy large breed dogs. We believe that the use of cCRP as a part of wellness screening would provide an early warning of occult disease, and prompt additional diagnostic testing. Given the findings presented here, it may be necessary to readjust the expected normal range. While our stated upper limit of normal was 7 U L^{-1} based on standard population based reference interval, this does not necessarily imply that this concentration is normal. In fact most values were quite low in healthy dogs, and that is why statistically the optimal cut-off value was determined to be 3.8 U L^{-1}.

The large sample size and prolonged follow-up are strengths of this study. One limitation is the relatively small number of diseased dogs. However, this is expected given our study design. The goal of this study was to develop a biomarker based screening test to detect occult disease. Most clinically healthy dogs will not be diseased so we anticipated a small number of affected dogs. Another limitation is the lack of additional evaluation at the time of sampling. In order to enrol the large sample size, it was not feasible to perform bloodwork and thoracic and abdominal imaging in all dogs, and the questionnaires were used to establish health. Because lymphoma and hemangiosarcoma are the two cancers for which TK1 can be markedly increased, it is unlikely that either of these cancers was occult for more than 6 months. Additionally, in each dog that was retested at the time of cancer diagnosis, the biomarkers had increased further. Some of the tumours detected (4 of 11) were not expected tumour types, suggesting a greater role for this test when screening for solid tumours as well. This also means that more prolonged follow-up and thorough evaluation will be needed to confirm true-negative subjects for calculation of specificity and sensitivity. For dogs that died of other causes, the lack of necropsy data limits our ability to confirm whether occult neoplasia could have been present. It should also be noted that dogs included in this study were either German shepherds or golden retrievers, so though breed-related differences are not expected, these data may not apply to all dogs.

In conclusion, the measurement of serum TK1 and cCRP, in combination as a NI, could be an effective method for screening for canine cancer.

The integration of these two biomarkers into an algorithm was more effective than either alone in identifying dogs with occult disease. Biomarkers are not designed or intended to be used without integration of clinical information, rather they are more powerful when used in combinations and when interpreted in light of clinical findings. On the basis of these findings, it is reasonable to consider second-wave diagnostics in an apparently healthy dog with increased TK1 or cCRP. Future studies should include prolonged follow-up and repeated sampling at regular and frequent intervals, in conjunction with clinicopathologic evaluation.

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Conflict of interest

Dr Selting is a paid consultant to VDI, and Randy Ringold is employed by VDI.

References

1. Nakamura N, Momoi Y, Watari T, Yoshino T, Tsujimoto H and Hasegawa A. Plasma thymidine kinase activity in dogs with lymphoma and leukemia. *The Journal of Veterinary Medical Science* 1997; **59**: 957–960.
2. von Euler H, Einarsson R, Olsson U, Lagerstedt AS and Eriksson S. Serum thymidine kinase activity in dogs with malignant lymphoma: a potent marker for prognosis and monitoring the disease. *Journal of Veterinary Internal Medicine* 2004; **18**: 696–702.
3. Madewell BR. Editorial: serum thymidine kinase activity: an alternative to histologic markers of cellular proliferation in canine lymphoma. *Journal of Veterinary Internal Medicine* 2004; **18**: 595–596.
4. von Euler H, Ohrvik AB and Eriksson SK. A non-radiometric method for measuring serum thymidine kinase activity in malignant lymphoma in dogs. *Research in Veterinary Science* 2006; **80**: 17–24.
5. von Euler H, Rivera P, Aronsson AC, Bengtsson C, Hansson LO and Eriksson SK. Monitoring therapy in canine malignant lymphoma and leukemia with serum thymidine kinase 1 activity - evaluation of a new, fully automated non-radiometric assay. *International Journal of Oncology* 2009; **34**: 505–510.
6. Thamm D, Kamstock DA, Sharp CR, Johnson SI, Mazzaferro E, Herold LV, Barnes SM, Winkler K and Selting KA. Elevated serum thymidine kinase activity in canine splenic hemangiosarcoma. *Veterinary and Comparative Oncology* 2012; **10**: 292–302.
7. Yamamoto S, Shida T, Miyaji H, Santsuka H, Fujise H, Mukawa K, Furukawa E, Nagae T and Naiki M. Changes in serum C-reactive protein levels in dogs with various disorders and surgical traumas. *Veterinary Research Communications* 1993; **17**: 85–93.
8. Yamamoto S, Shida T, Okimura T, Otabe K, Honda M, Ashida Y, Furukawa E, Sarikaputi M and Naiki M. Determination of C-reactive protein in serum and plasma from healthy dogs and dogs with pneumonia by ELISA and slide reversed passive latex agglutination test. *The Veterinary Quarterly* 1994; **16**: 74–77.
9. Ceron JJ, Eckersall PD and Martynez-Subiela S. Acute phase proteins in dogs and cats: current knowledge and future prospectives. *Veterinary Clinical Pathology* 2005; **34**: 85–99.
10. Nielsen L, Toft N, Eckersall PD, Mellor DJ and Morris JS. Serum C-reactive protein concentration as a indicator of remission status in dogs with multicentric lymphoma [abstract]. *Journal of Veterinary Internal Medicine* 2007; **21**: 1231–1236.
11. Mischke R, Waterston M and Eckersall PD. Changes in C-reactive protein and haptoglobin in dogs with lymphatic neoplasia. *Veterinary Journal* 2007; **188**: 192.
12. Nakamura M, Takahashi M, Ohno K, Koshino A, Nakashima K, Setoguchi A, Fujino Y and Tsujimoto H. C-reactive protein concentration in dogs with various diseases. *The Journal of Veterinary Medical Science* 2008; **70**: 127–131.
13. Sheahan D, Bell R, Mellanby RJ, Gow AG, Friend E, Heller J, Bence LM and Eckersall PD. Acute phase protein concentrations in dogs with nasal disease. *The Veterinary Record* 2010; **167**: 895–899.
14. Dabrowski R, Kostro K, Lisiecka U, Szczubiał M and Krakowski L. Usefulness of C-reactive protein, serum amyloid A component, and haptoglobin determinations in bitches with pyometra for monitoring early post-ovariohysterectomy complications. *Theriogenology* 2009; **72**: 471–476.
15. Xu XH, Zhang YM, Shu XH, Shan LH, Wang ZW, Zhou YL, Wen HK, He F, He E and Skog S. Serum thymidine kinase 1 reflects the progression of

premalignant and malignant tumors during therapy. *Molecular Medicine Reports* 2008; **1**: 705–711.
16. Chen Y, Ying M, Chen Y, Hu M, Lin Y, Chen D, Li X, Zhang M, Yun X, Zhou J, He E and Skog S. Serum thymidine kinase 1 correlates to clinical stages and clinical reactions and monitors the outcome of therapy of 1,247 cancer patients in routine clinical settings. *International Journal of Clinical Oncology* 2010; **15**: 359–368.
17. Elliott JW, Cripps P and Blackwood L. Thymidine kinase assay in canine lymphoma. *Veterinary and Comparative Oncology* 2011; **11**: 1–13.
18. Hannigan BM, Barnett YA, Armstrong DBA, McKelvey-Martin VJ and McKenna PG. Thymidine kinases: the enzymes and their clinical usefulness. *Cancer Biotherapy* 1993; **8**: 189–197.
19. Tsilidis KK, Branchini C, Guallar E, Helzlsouer KJ, Erlinger TP and Platz EA. C-reactive protein and colorectal cancer risk: a systematic review of prospective studies. *International Journal of Cancer* 2008; **123**: 1133–1140.
20. Qian BZ and Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell* 2010; **131**: 39–51.
21. Rakoff-Nahoum S. Cancer mechanisms: why cancer and inflammation? *The Yale Journal of Biology and Medicine* 2006; **79**: 123–130.
22. Hanahan D and Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646–674.
23. Coussens LM and Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860–867.
24. Chen CC, Chang TW, Chen FM, Hou MF, Hung SY, Chong IW, Lee SC, Zhou TH and Lin SR. Combination of multiple mRNA markers (PTTG1, survivin, UbcH10 and TK1) in the diagnosis or Taiwanese patients with breast cancer by membrane assay. *Oncology* 2006; **70**: 438–446.
25. Connolly Y, Littler E, Sun N, Chen X, Huang PC, Stacey SN and Arrand JR. Antibodies to Epstein-Barr virus thymidine kinase: a characteristic marker for the serological detection of nasopharyngeal carcinoma. *International Journal of Cancer* 2001; **91**: 692–697.
26. Jasensky JK, Hennies M, Kohn B, Einspanier R. Development of an Enzyme-linked immunosorbent assay (ELISA) for the measurement of canine C-reactive protein. Poster and abstract, InnLab Conference 2012, Gottingen, Germany, February 2–3, 2012.
27. Chen ZH, Huang SQ, Wang Y, Yang AZ, Wen J, Xu XH, Chen Y, Chen QB, Wang YH, He E, Zhou J and Skog S. Serological thymidine kinase 1 is a biomarker for early detection of tumours – a health screening study on 35,365 people, using a sensitive chemiluminescent dot blot assay. *Sensors* 2011; **11**: 11064–11080.

