

# Plasma procalcitonin concentrations are increased in dogs with sepsis

Robert Goggs,<sup>1</sup> Matthew Milloway,<sup>1</sup> Roberta Troia,<sup>2</sup> Massimo Giunti<sup>2</sup>

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## ABSTRACT

Sepsis, the life-threatening organ dysfunction caused by a dysregulated host response to infection, is difficult to identify and to prognosticate for. In people with sepsis, procalcitonin (PCT) measurement aids diagnosis, enables therapeutic monitoring and improves prognostic accuracy. This study used a commercial canine PCT assay to measure plasma PCT concentrations in dogs with gastric dilatation volvulus (GDV) syndrome and in dogs with sepsis. It was hypothesised that dogs with GDV syndrome and with sepsis have greater plasma PCT concentrations than healthy dogs and that dogs with sepsis have greater PCT concentrations than dogs with GDV syndrome. Before analysing canine plasma samples, the ability of the assay to identify canine PCT, in addition to assay imprecision and the lower limit of detection were established. The assay had low imprecision with coefficients of variation  $\leq 4.5$  per cent. The lower limit of detection was 3.4 pg/ml. Plasma PCT concentrations were measured in 20 dogs with sepsis, in 32 dogs with GDV syndrome and in 52 healthy dogs. Median (IQR) PCT concentration in dogs with sepsis 78.7 pg/ml (39.1–164.7) was significantly greater than in healthy dogs 49.8 pg/ml (36.2–63.7) ( $P=0.019$ ), but there were no significant differences between PCT concentrations in dogs with GDV syndrome and controls ( $P=0.072$ ) or between dogs with sepsis and GDV syndrome ( $P=1.000$ ). Dogs with sepsis have significantly increased plasma PCT concentrations compared with healthy dogs, although considerable overlap between these populations was identified. Future investigations should confirm this finding in other populations and evaluate the diagnostic and prognostic value of PCT in dogs with sepsis.

## INTRODUCTION

Sepsis was recently redefined as the life-threatening organ dysfunction caused by a dysregulated host response to infection.<sup>1</sup> Sepsis can be difficult to diagnose and to differentiate from other causes of systemic inflammation. It can also be challenging to provide an accurate prognosis for patients with sepsis. Biomarkers may aid clinicians with these challenges, and many have been investigated in human and canine sepsis.<sup>2</sup> In people with sepsis, procalcitonin (PCT) has been repeatedly demonstrated to aid diagnosis, enable monitoring of response to therapy and improve prognostic accuracy. In humans, increased blood PCT concentrations are used to differentiate bacterial sepsis from non-infectious systemic

inflammatory response syndrome (SIRS).<sup>3</sup> In human sepsis, PCT levels correlate with disease severity, and predict mortality.<sup>4,5</sup> Measurement of PCT may be used to guide both the initiation and discontinuation of antimicrobial therapy.<sup>6–9</sup>

PCT is the precursor of the hormone calcitonin. In health, PCT is produced by the parafollicular (C) cells in the thyroid gland and converted to calcitonin in response to increased plasma calcium concentrations.<sup>10,11</sup> In healthy people, plasma PCT concentrations are typically below the level of detection of clinical assays ( $<10$  pg/ml).<sup>12</sup> PCT is regulated differently during sepsis than in health.<sup>13</sup> In sepsis, PCT concentrations increase in response to an infectious stimulus and peak at 24 hours,<sup>3</sup> and likely originate from monocytes, neutrophils and liver, kidney, spleen and lung. The secretion of PCT by monocytes can occur in response to endotoxin and cytokine stimulation,<sup>14</sup> and is tightly regulated, such that it may require both pathogen recognition and cell-cell interactions.<sup>15,16</sup> PCT may inhibit chemotaxis,<sup>17</sup> induce cytokines<sup>18</sup> and affect vascular tone through antagonism of calcitonin gene-related peptide and adrenomedullin.<sup>19</sup> PCT also increases nitric oxide release and therefore may play a role in amplification of inflammation.<sup>20</sup>

To date, there is very little information regarding PCT in dogs. A gene expression study suggested that dogs with canine parvovirus infection had widespread expression of the Calcitonin-related Polypeptide Alpha (CALCA) gene in the spleen, lung and liver,<sup>21</sup> likely secondary to systemic inflammation. The canine CALCA gene (UNIPROT: Q9MYV1) encodes the peptide hormone calcitonin, but can be translated into two alternative peptide hormones, calcitonin gene-related peptide and katecalcitonin by alternative splicing and peptide cleavage. The principal roles of these peptide hormones are calcium regulation, host defence and neurotransmission. PCT mRNA is increased in circulating leucocytes collected from dogs with various critical illnesses including sepsis and cancer,<sup>22</sup>



<sup>1</sup>Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York City, USA

<sup>2</sup>Department of Veterinary Medical Sciences, Alma Mater Studiorum, University of Bologna, Bologna, Italy

### Correspondence to

Dr Robert Goggs; [r.goggs@cornell.edu](mailto:r.goggs@cornell.edu)

perhaps suggesting that PCT behaves as an acute phase protein in dogs as in horses.<sup>23</sup> It was reported that dogs with babesiosis have increased blood PCT concentrations,<sup>24</sup> but that study used an assay that does not reliably detect canine PCT, because the assay standard does not actually contain PCT protein.<sup>25</sup>

Recently, an alternative commercial PCT ELISA assay has become available. The aims of the present study were therefore (i) to confirm the identity of a recombinant PCT protein and the assay's protein standard using mass spectrometry (MS); (ii) to confirm that the assay detects canine PCT; (iii) to use the assay to measure canine PCT in plasma samples collected from dogs with sepsis and gastric dilatation volvulus (GDV) syndrome. The authors hypothesised that dogs with GDV syndrome and with sepsis have greater plasma PCT concentrations than healthy dogs and that dogs with sepsis have greater PCT concentrations than dogs with GDV syndrome.

## MATERIALS AND METHODS

### Mass spectrometry protein identification

Purified, recombinant canine PCT (produced in *Escherichia coli*) was purchased from a commercial source (ab188456, Abcam, Cambridge, Massachusetts, USA). This protein, and the protein standard from the commercial ELISA kit were submitted to the institution's core facility for protein identification by MS. Samples were prepared individually for protein digestion, peptide labelling and identification. The estimated protein mass in each sample was 5 µg. Proteins were solubilised with 6 M guanidine hydrochloride and 50 mM tris (both Fisher Chemical, Waltham, Massachusetts, USA) and reduced with 110 mM dithiothreitol (Roche, Basel, Switzerland), gently vortexed, centrifuged and incubated at 60°C for one hour. Samples were then alkylated with 50 mM iodoacetamide (Acros Organics, Geel, Belgium), incubated for 30 minutes in the dark at room temperature and then quenched with additional 0.5 M dithiothreitol. The solutions were then diluted with 50 mM ammonium bicarbonate (Fisher Chemical) and digested with 0.2 µg/µl trypsin for 18 hours at 37°C. The reaction was then quenched by addition of 100 per cent formic acid (Fisher Chemical) and samples dried in a speed vacuum centrifuge. The samples were then desalted using SOLA HRP solid-phase extraction cartridges (60109-003, Thermo Fisher Scientific, San Jose, California, USA) preconditioned with 90 per cent methanol (Fisher Chemical) and 0.1 per cent trifluoroacetic acid (Fluka, St. Louis, Missouri, USA), eluted with 50 per cent acetonitrile (ACN). To remove possible detergent contamination, samples were run through Oasis MCX mixed-mode polymeric sorbent cartridges (Waters Corporation, Milford, Massachusetts, USA) or cation exchange cartridges<sup>1</sup> preconditioned with 100 per cent methanol and 10 mM hydrochloric acid (Fisher Chemical), washed with 95 per cent acetonitrile, 5 mM HCl and eluted with 10 per cent ammonium hydroxide,

75 per cent ACN. The liquid chromatography tandem MS analyses were conducted using a UltiMate3000 nanoLC device (Dionex, Sunnyvale, California, USA) coupled to an Orbitrap Fusion tandem mass spectrometer (Thermo Fisher Scientific) equipped with a nanospray ion source. Samples were reconstituted in 2 per cent acetonitrile and 0.5 per cent formic acid and loaded onto an Acclaim PepMap 100 C18 trap column (Thermo Fisher Scientific) for desalting followed by peptide separation using an Acclaim PepMap C18 nano column (75 µm x 25 cm, Thermo Fisher Scientific).

The orbitrap mass spectrometer was operated in positive ion mode with nanospray voltage set at 1.5 kV and source temperature at 275°C. The orbitrap full MS survey scan ( $m/z$  375–1575) was followed by top three-second data-dependent collision-induced dissociation MS/MS scans for precursor peptides with two to seven charges above a threshold ion count of 5000 with normalised collision energy of 30 per cent. MS survey scans were acquired at a resolving power of 120 000 ( $m/z$  200), with automatic gain control (AGC) 4e5 and maximum injection time (IT) 50 ms. Tandem MS scans were acquired with AGC 1e4, max IT 70 ms and with isolation window ( $m/z$ ) at 1.6 for the mass range  $m/z$  110–2000. Dynamic exclusion parameters were set at 1 within 40 seconds exclusion duration with ±10 ppm exclusion mass width. All data were acquired using proprietary software (Xcalibur 2.0 operation software, Orbitrap Fusion Tune Application V.2.0, Thermo Fisher Scientific). All MS and MS/MS raw spectra from each sample were searched using proprietary software (Proteome Discoverer V.1.4, Thermo Fisher Scientific) and an online protein database (<http://www.uniprot.org/>) for *E. coli* containing 4274 sequences, including targeted proteins. The peptide search was performed using a proprietary algorithm (Sequest HT Proteome Discoverer V.1.4), allowing two missed cleavage for full trypsin digestion, fixed carbamidomethyl modification of cysteine and variable oxidation of methionine and deamidation of asparagine/glutamine residues. The peptide mass tolerance and fragment mass tolerance values were 10 ppm and 0.6 Da, respectively. Identified peptides were filtered for maximum 1 per cent false discovery rate and minimum peptide confidence set to high. Results of the search were exported by the software as a spreadsheet.

### ELISA kit protocol

PCT concentrations in all patient and control samples were measured in duplicate and the mean value used for subsequent analyses. No samples were reanalysed due to high coefficients of variation (CV) values. The canine PCT ELISA kit (Biovendor, Asheville, North Carolina, USA) was used per the manufacturer's instructions with slight modifications. The manufacturer recommends diluting samples fivefold before measurement. This was attempted initially but the resulting absorbance values for control dogs were commonly below the readable range of the assay (data not shown). All subsequent assays were

performed using undiluted samples. The manufacturer recommends use of an automatic plate washer, but this apparatus was not available and hence manual washing was performed using 275 µl per well (instead of the recommended 350 µl), with the addition of a fourth wash at each wash step. All assays were read using a Synergy HI Hybrid Multi-Mode microplate reader (Bio-Tek, Winooski, Vermont, USA), set per the kit manufacturer's instructions and the data analysed using proprietary software (Gen5 Microplate Reader and Imager Software, Bio-Tek). Standard curves were constructed by duplicate measurement of the supplied protein standard, diluted to concentrations 12.5, 25, 50, 100, 200, 400 and 800 pg/ml, per the manufacturer's recommendations. Standard curves were generated by fitting a 4-parameter model ( $Y=(A-D)/(1+(X/C)^B)+D$ ) using an iterative series of calculations using the Newton-Raphson algorithm to determine the best curve-fit by the least squares method. All standard curves were manually inspected to ensure that the curve-fitting model consistently had  $R^2$  values of 1.

### Positive control testing

The ability of the kit to positively identify PCT was first tested using a recombinant canine PCT protein (ab188456, Abcam). This protein was reconstituted according to the supplier's instructions and a dilution series (6.25–8000 pg/ml) constructed in PBS.

### ELISA kit imprecision and lower limit of detection

To assess imprecision, samples of the kit dilution buffer spiked to three known final concentrations of PCT (50, 200 and 400 pg/ml) were generated using the kit manufacturer's supplied PCT protein standard. These samples were each then repeatedly analysed ( $n=20$ ) and the mean, sd and CV calculated. The lower limit of the blank (LoB) was established as the mean blank value  $+1.645 \times sd_{\text{blank}}$  per Armbruster and Pry.<sup>26</sup> The mean blank value was estimated by repeated measurement ( $n=20$ ) of the blank (assay kit sample buffer). The lower limit of detection (LoD) was established as  $LoB+1.645 \times sd_{\text{low conc}}$ . Data from repeated measurement of the low concentration sample in the spike/recovery experiments were used to provide the low concentration sd value.

### Canine plasma sample analysis

Canine plasma samples were collected from patients managed at the two participating locations (Cornell University, Ithaca, New York, USA and University of Bologna, Italy). Specifically, stored citrate plasma samples collected from 30 dogs with GDV syndrome (Cornell  $n=20$ , Bologna  $n=12$ ), and 20 dogs with sepsis (Cornell  $n=20$ ) enrolled as part of other studies were analysed. After centrifugation and separation, samples were frozen at  $-80^\circ\text{C}$  until the time of analysis. The longest duration of storage before analysis was 18 months. All dogs were sampled at the time of presentation to the recruiting institutions. Those studies were approved by

the local Institutional Animal Care and Use Committee (IACUC) and undertaken with written informed client consent (Cornell IACUC 2014–0053; Bologna DL 26/2014, Project 581). The diagnosis of bacterial sepsis was based on the 2001 Society of Critical Care Medicine consensus definition for sepsis<sup>27</sup> and used established canine SIRS criteria,<sup>28</sup> combined with the presence of highly suspected or confirmed bacterial infection. Specifically, the SIRS criteria employed were as follows: rectal temperature less than  $38^\circ\text{C}$  or greater than  $39^\circ\text{C}$ ; heart rate greater than 120 bpm; respiratory rate greater than 20 rpm; total leucocyte count less than  $6 \times 10^9/\text{L}$  or greater than  $16 \times 10^9/\text{L}$ , or the presence of more than 3 per cent band neutrophils.<sup>28</sup> Dogs with bodyweight less than 3 kg, prolonged clotting times above the reference intervals or thrombocytopenia less than  $30 \times 10^9/\text{L}$  were excluded. The diagnosis of GDV syndrome was based on compatible clinical signs and the presence of characteristic gas distension and displacement of the stomach on right lateral abdominal radiographs.<sup>29</sup> Fifty-two healthy privately owned dogs weighing greater than 3 kg were enrolled as controls (Cornell  $n=40$ , Bologna  $n=12$ ), again with local IACUC approval (Cornell IACUC 2014–0052; Bologna DL 26/2014, Project 581). Control dogs were eligible for inclusion if they had no history or evidence of recent or chronic medical conditions and had not received any medication, except for routine preventative healthcare, within the preceding 3 months. Dogs were classified healthy on the basis of history, physical examinations and complete blood count and serum chemistry results.

### Statistical analysis

Before test selection, data were assessed for normality by assessment of histograms, calculation of skewness and kurtosis and with the D'Agostino Pearson test and descriptive statistics calculated as appropriate. Parametric data are presented as  $\text{mean} \pm \text{sd}$ , non-parametric data are presented as median (IQR). CVs were calculated as  $\text{mean}/\text{sd}$ . Procalcitonin concentrations in the healthy control and patient samples were not normally distributed. Thus, PCT concentrations were compared between dogs with sepsis, GDV syndrome and controls by Kruskal-Wallis with Dunn's post hoc multiple comparisons test. These analyses were performed using commercial software (Prism V.7.0, GraphPad, La Jolla, California, USA). Alpha was set at 0.05. A reference interval (RI) for PCT was calculated from the healthy control data according to previous published recommendations.<sup>30</sup> Briefly, healthy control data ( $n=52$ ) were analysed for outliers by Tukey's method. One outlier was identified and removed before RI generation using robust methods to calculate the RI bounds and their 90 per cent CIs. These CIs were estimated by bootstrapping according to the percentile interval method,<sup>31</sup> using 10 000 iterations and a random-number seed of 928. Outlier identification and RI calculation was performed using commercial software (MedCalc 17.8.5, MedCalc Software, Ostend, Belgium).



**TABLE 1:** Summary data from tandem mass spectrometry identifications

Sample	Accession	Description	Score	Coverage	Unique peptides	PSMs
Recombinant PCT protein	P41547	Calcitonin <i>Canis lupus familiaris</i> Gene name CALCA	4618.25	98.26	25	1520
PCT ELISA standard	P41547	Calcitonin <i>Canis lupus familiaris</i> Gene name CALCA	4.00	17.39	1	1

CALCA, Calcitonin-related Polypeptide Alpha; PCT, procalcitonin; PSMs, peptide spectrum matches.

## RESULTS

### Mass spectrometry analysis

In the sample of recombinant canine procalcitonin, the targeted protein was the strongest identification based on the protein score (online supplementary file 1). Canine procalcitonin was identified in the ELISA kit assay standard, although it was not the protein with the strongest identification in that analysis (online supplementary file 2). The strongest identification from the ELISA standard was for a contaminating *E coli* protein (phosphoglycerate kinase, accession P0A799). The protein sequence coverage, protein scores and number of peptide sequence matches (PSMs) were low for PCT in both samples (Table 1). The protein score reported by the MS analysis is the sum of the cross-correlation scores (XCORR) of individually identified peptides compared with the reference protein database. The PSM values for both proteins were low. The PSM represents the total number of identified peptide sequences for the protein, including those redundantly identified. The protein sequence coverage is a value calculated by dividing the number of amino acids in all found peptides by the total number of amino acids in the entire protein sequence. Essentially, the PSM value is a score that expresses the likelihood that the experimentally identified spectrum is derived from a specific, known protein. Hence, the higher the PSM value the more likely the sample contains the identified protein. The low PSM values, coupled with low sequence coverage and the low peptide XCORR values likely indicates an overall low abundance of PCT in the samples submitted for identification. The total number of identified proteins in both samples was low, however, which indicates that both samples were of good purity.

### Positive control

The PCT assay kit positively identified the recombinant PCT protein in a linear, concentration-dependent manner ( $R^2=0.974$ ,  $P<0.0001$ ) (online supplementary file 3), although calculated concentrations  $\geq 400$  pg/ml of the recombinant protein were required to generate positive test results.

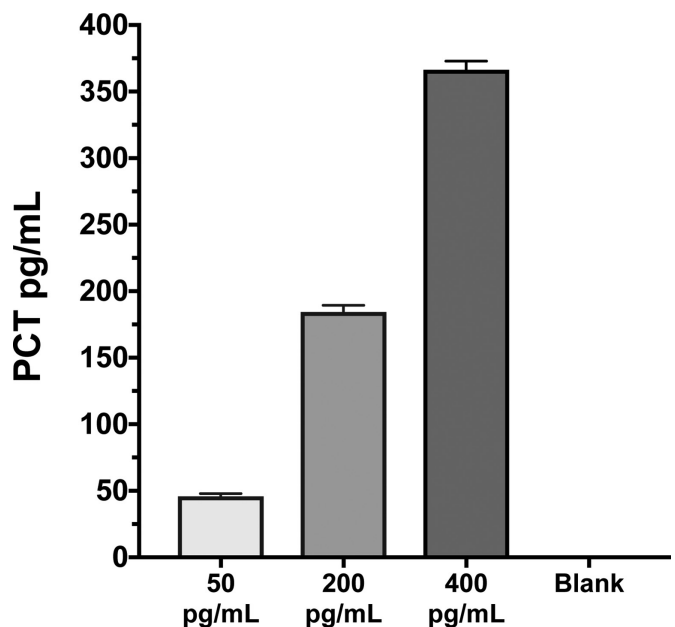
### Imprecision and limit of detection

CVs for the low, medium and high spike concentrations of the diluent buffer were 4.5, 2.7 and 1.8 per cent, respectively (Fig 1). A CV could not be calculated for the repeated measurements of the blank because all of

the values were 0. The mean $\pm$ sd value for the lower limit of the blank was 0 pg/ml $\pm$ 0. This established a value for the LoB of 0 pg/ml. The sd for the low concentration repeated measurement was 2.1. This established a value for the LoD of 3.4 pg/ml.

### Canine plasma sample analysis

Twenty dogs with sepsis were enrolled. There were seven intact female dogs, six spayed female dogs, four castrated male dogs and three intact male dogs. The mean age was 6.4 $\pm$ 2.9 years. The mean bodyweight was 33.8 $\pm$ 14.9 kg. All dogs met at least two/four SIRS criteria.<sup>28</sup> The mean rectal temperature on presentation was 39.6 $\pm$ 0.8°C, the mean heart rate was 134 $\pm$ 32 bpm, the mean respiratory rate was 41 $\pm$ 14 bpm and the median leucocyte count was 17.6 $\times 10^9$ /L (9.9–25.6). The causes of sepsis were pyometra (n=6), septic peritonitis (n=5), pneumonia



**FIGURE 1:** Assay repeatability and spike/recovery were evaluated by repeated analysis (all n=20) of samples spiked to known final concentrations of procalcitonin. The diluent buffer supplied by the manufacturer was spiked to three concentrations of procalcitonin (PCT) (50, 200, 400 pg/ml) using the protein standard supplied in the kit. Repeated measurement (n=20) of the diluent buffer was used to establish the lower limit of the blank. All blank PCT concentrations were 0. Bars represent mean, error bars represent sd.

(n=4), abscess (n=4) and osteomyelitis (n=1). Nine dogs had positive blood cultures. Fourteen dogs (70 per cent) survived to hospital discharge. All six non-survivors were euthanased for disease severity, development of life-threatening complications or perceived poor prognosis. No dogs were euthanased for financial limitations.

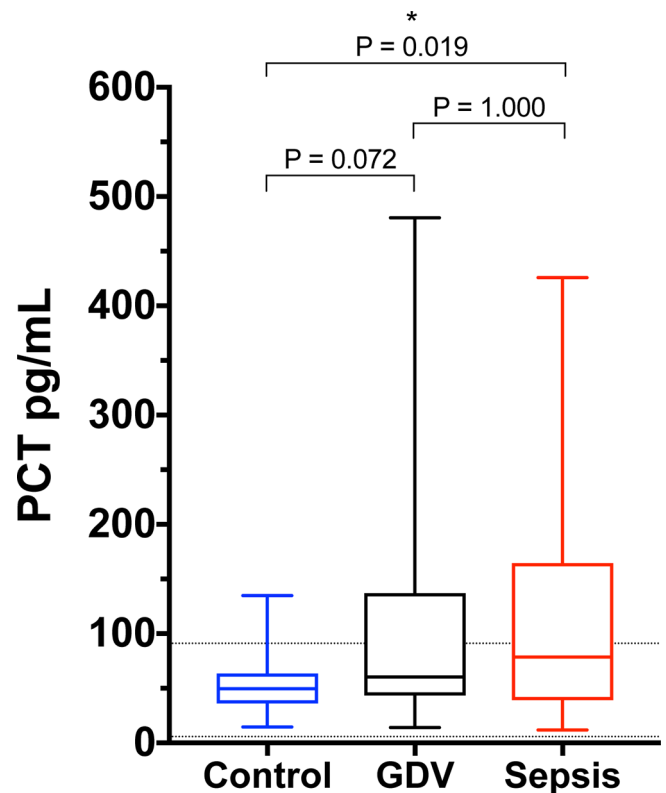
Thirty-two dogs with GDV syndrome were enrolled. There were 12 castrated male dogs, 9 intact male dogs, 8 spayed female dogs and 3 intact female dogs. The mean age was  $8.4 \pm 3.8$  years. The mean bodyweight was  $39.1 \pm 11.5$  kg. All dogs met at least two/four SIRS criteria.<sup>28</sup> The mean rectal temperature on presentation was  $38.3 \pm 1.0^\circ\text{C}$ , the mean heart rate was  $158 \pm 38$  bpm, the median respiratory rate was 40 bpm (36–60) and the mean leucocyte count was  $15.3 \times 10^9/\text{L} \pm 5.5$ . Twenty-four dogs (75 per cent) survived to hospital discharge. All eight non-survivors were euthanased for disease severity, development of life-threatening complications or perceived poor prognosis. No dogs were euthanased for financial limitations. Compared with dogs with sepsis, dogs with GDV syndrome were significantly older ( $P=0.041$ ), had significantly lower rectal temperatures ( $P<0.0001$ ) and higher heart rates ( $P=0.016$ ). There was no significant difference in bodyweight, respiratory rate or leucocyte counts between dogs with sepsis and those with GDV syndrome.

The healthy control dog population (n=52) consisted of 24 spayed female dogs, 12 castrated male dogs, 9 intact female dogs and 7 intact male dogs. The mean age was  $5.0 \pm 2.9$  years. The mean bodyweight was  $24.9 \pm 11.7$  kg.

The calculated RI for canine PCT was 5.8–91.1 pg/ml, with 90 per cent CI for the lower bound of 0.0–14.3 pg/ml and for the upper bound of 82.1–101.1 pg/ml. Using the whole healthy control population (n=52), the median PCT concentration in the dogs with sepsis 78.7 pg/ml (39.1–164.7) was significantly greater than in the healthy control dogs 49.8 pg/ml (36.2–63.7) ( $P=0.019$ ). There was no significant difference between the median PCT concentrations in dogs with GDV syndrome (60.3 pg/ml (43.3–137.2)) compared with healthy controls ( $P=0.072$ ) or between dogs with sepsis and dogs with GDV syndrome ( $P=1.000$ ) (Fig 2). Sample CV values were non-parametric. The median (IQR) CV for all samples was 3.4 per cent (1.8–6.7).

## DISCUSSION

The present study confirmed that a commercial PCT ELISA detects canine PCT, has low imprecision and an acceptable lower LoD and demonstrates that dogs with sepsis have significantly increased PCT compared with healthy controls, but dogs with GDV syndrome do not. The present study aimed to confirm that the Biovendor canine PCT ELISA assay was able to detect canine procalcitonin and to use it to determine plasma PCT concentrations in dogs with sepsis and GDV syndrome. A previous publication evaluating a different commercial PCT ELISA<sup>25</sup> found that the assay standard did not contain



**FIGURE 2:** Box and whisker plot comparison of plasma procalcitonin (PCT) concentrations from 40 healthy control dogs, 20 dogs with gastric dilatation volvulus (GDV) syndrome and 20 dogs with sepsis. The central line represents the median, the boxes represent the IQR and the whiskers represent the minimum and maximum values. Dogs with sepsis had significantly greater plasma concentrations of PCT compared with healthy controls ( $P=0.019$ ) by Kruskal-Wallis with Dunn's post hoc multiple comparisons test, indicated by an asterisk (\*). Horizontal dotted lines represent the reference intervals 9.4–93.3 pg/ml calculated by the robust method, wherein the CIs were estimated by bootstrapping according to the percentile interval method (Efron and Tibshirani), using 10 000 iterations and a random number seed of 928.

PCT and hence the readings from that assay could not be relied on. The first step in the present study was therefore to confirm that the Biovendor assay could identify canine PCT. To do this, the manufacturer's protein standard and a separate recombinant canine PCT protein were tested for the presence of canine PCT by MS. Both the manufacturer's standard and the recombinant protein contained pure canine PCT albeit at low concentrations.

The recombinant canine PCT protein used as a positive control was detected by the assay, confirming that the assay is capable of measuring canine PCT. However, the concentrations of protein measured with the ELISA were lower than the theoretical concentrations calculated per the dilution series. There are several potential explanations for this. First, the recombinant protein may have been slightly distinct from the target used to raise the capture antibody, perhaps not sufficient to prevent binding, but sufficient to reduce assay sensitivity. This

may have resulted from the protein being generated in *E coli* rather than mammalian cells, since the PCT protein is glycosylated. Second, although the recombinant protein was shipped lyophilised, it is possible that some degradation of the protein occurred before use or during reconstitution, thereby reducing the real protein concentration. Third, the diluent used to generate the dilution series may have interfered with the assay. The protein was reconstituted in purified water and then diluted into PBS and the use of a non-protein buffer may have impacted the stability of the recombinant protein or impaired the interaction with the assay's capture antibody. Use of the manufacturer supplied buffer or spiking of the recombinant protein into canine plasma might have provided a more suitable matrix and improved the correlation between calculated and measured concentrations.

Imprecision testing determined that the assay has low CVs (all CVs  $\leq 4.5$  per cent), similar to the values reported by the assay manufacturer (within-run CV 4.6 per cent at 242 pg/ml, 3.7 per cent at 528.5 pg/ml). The measured values for the PCT concentrations in these experiments were all  $\sim 8$  per cent lower than the expected concentrations. This loss of recovery was consistent across the concentrations and hence, it is unlikely that this would have a meaningful effect on comparisons between measurements from cases and controls. The repeated measurement of the blank and the low concentration spiked samples enabled the establishment of an LoD for the assay. This value is low (3.4 pg/ml), which suggests that the assay is capable of discriminating low concentrations of PCT from background.

On the basis of human data, and from other investigations in dogs it was hypothesised that dogs with sepsis and dogs with GDV syndrome have increased plasma PCT concentrations. The results of the present study support the hypothesis that dogs with sepsis have increased plasma PCT concentrations, but suggest that dogs with GDV syndrome do not have increased PCT concentrations relative to healthy controls. These data are somewhat consistent with the literature. Two previous studies have shown that mRNA for the CALCA gene is expressed in various tissues in dogs with sepsis and with other inflammatory conditions.<sup>21 22</sup> A separate study suggested that blood PCT concentrations are increased in dogs with experimentally induced endotoxemia,<sup>32</sup> however human assays may not differentiate PCT values in dogs with SIRS from those in healthy controls.<sup>33</sup> Dogs with GDV syndrome are recognised to be commonly affected by SIRS,<sup>34 35</sup> and hence were studied here to determine if PCT concentrations were able to distinguish non-infectious SIRS from sepsis. This choice of comparative population is not without limitation, however, since dogs with GDV syndrome can develop sepsis due to bacterial translocation or aspiration pneumonia. These conditions were not identified in the GDV syndrome dogs studied, but could have been present subclinically. All dogs in the present study were sampled at the time of presentation, but likely had varying durations of symptoms

before hospital admission. Additionally, although dogs with GDV syndrome were studied as a population of dogs at-risk for SIRS, the SIRS criteria can easily be satisfied in dogs with GDV by tachycardia associated with shock and through respiratory compromise secondary to abdominal distension. The data from the present study could be interpreted as indicating that PCT concentrations in dogs increase in response to pathogen interactions. However, the potential confounding from occult pathogen-host interactions in the dogs with GDV suggest that PCT may be acting as an acute phase reactant—responding to conditions associated with inflammation. Further studies in additional populations of dogs with sepsis and with other conditions causing systemic inflammation will be necessary to answer this question.

In human medicine, PCT measurements are discriminating for patients with infection.<sup>3</sup> In the present study, there was considerable overlap in the measured PCT concentrations between healthy controls and the dogs with sepsis. Comparison with the calculated reference intervals also suggests that most of the patients with sepsis included in the present study would have been classified as 'normal'. Although the present study detected a significant difference between PCT concentrations in dogs with sepsis compared with healthy controls, the degree of overlap between the measured concentrations is likely to substantially limit the utility of PCT measurements for the diagnosis of sepsis in dogs. Interestingly, there was considerable variation in the PCT concentrations within the sepsis population, with a 36-fold difference between the lowest and the highest measured concentration (absolute difference 414 pg/ml). This range suggests that PCT measurements might be able to distinguish subpopulations of dogs with sepsis with different levels of illness severity. Although PCT may be of limited value for the diagnosis of sepsis in dogs, the authors speculate that it might be of value for assessment of prognosis. As such, further investigation of associations between PCT, illness severity and outcome in canine sepsis appears warranted.

In conclusion, the present study suggests that the canine PCT ELISA employed here can be used to measure plasma PCT concentrations in dogs and that dogs with sepsis have increased concentrations of PCT compared with healthy controls. Future investigations might be directed to confirming these findings in other sepsis populations, to correlating PCT with other biomarkers and illness severity assessments, to measuring the kinetics of PCT concentrations over time and to evaluating the prognostic value of PCT in dogs with sepsis.

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manuscript revision, RT performed assays, data analysis and manuscript revision, MG assisted with study design, data interpretation and manuscript revision. All authors approved the final version of the manuscript.

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