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STANDARD ARTICLE

Serial analysis of blood biomarker concentrations in dogs with pneumonia, septic peritonitis, and pyometra

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Abstract

Background: Prolonged antimicrobial drug (AMD) treatment is associated with antimicrobial resistance development. Biomarker measurement may aid treatment decision-making.

Objectives: Investigate temporal changes in blood biomarker concentrations in dogs undergoing treatment for pulmonary and intra-abdominal infections; compare time to biomarker concentration normalization with duration of clinician-directed AMD treatment.

Animals: Forty-two client-owned dogs with pneumonia (n = 22), septic peritonitis (n = 10), or pyometra (n = 10).

Methods: Plasma concentrations of C-reactive protein (CRP), serum amyloid A (SAA), haptoglobin, procalcitonin, nucleosomes, cell-free DNA (cfDNA), high-mobility group box-1 (HMGB1), CC-motif chemokine ligand-2 (CCL2), CXC-motif chemokine ligand-8 (CXCL8), and keratinocyte chemoattractant-like (KC-Like) were quantitated in samples collected on days 1, 3, 7, 14, 28, and 60. Treatment was directed by clinicians blinded to biomarker concentrations.

Results: Concentrations of CCL2, CRP, and KC-Like were maximal on D1, concentrations of SAA, cfDNA, HMGB1, and nucleosomes were maximal on D3 and haptoglobin concentrations were maximal on D7. These maximal concentrations were significantly different from those on D60. Concentrations of CRP and SAA decreased by 80% from peak and into respective reference intervals before AMDs were discontinued. For CRP, the median (interquartile range [IQR]) times to 20% peak and normal were 7 (6-9) and 7 (6-12) days, respectively, and for SAA they were 4 (4, 5) and 6 (5-8) days, respectively, compared to a median (IQR) duration of AMD prescribing of 16 (12-23) days (all $P < .0001$).

Conclusions and Clinical Importance: Biomarker concentrations normalized within 7 to 14 days. Serial measurements of CRP and SAA might aid identification of disease resolution and could help guide AMD prescription decision-making.

Abbreviations: APP, acute phase protein; APPLE, acute patient physiologic and laboratory evaluation; AU, arbitrary unit; CCL2, C-C motif chemokine ligand 2; cfDNA, cell-free DNA; CRP, C-reactive protein; CXCL8, C-X-C motif chemokine ligand 8; GDV, gastric dilatation-volvulus; HMGB1, high mobility group box-1; IMHA, immune-mediated hemolytic anemia; IQR, interquartile range; KC-like, keratinocyte chemoattractant-like; PCT, procalcitonin; RI, reference interval; SAA, serum amyloid A.

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KEYWORDS
acute phase proteins, CRP, cytokines, haptoglobin, HMGB1, procalcitonin, SAA

1 | INTRODUCTION

Sepsis in dogs with pneumonia, peritonitis, and pyometra is associated with mortality rates of 30% to 64%.[1,2] Early recognition and management consisting of cardiovascular stabilization, infection source control, and administration of appropriate antimicrobial drugs (AMDs) are essential to maximize survival in patients with sepsis.[3-6] Extensive AMD use however contributes to bacterial antimicrobial resistance (AMR) by exerting selection pressure.[7-10] In dogs, recent AMD exposure is associated with pneumonia caused by drug-resistant pathogens,[11] and with inappropriate empirical AMD selection for patients with septic peritonitis.[12] Shorter durations of AMD treatment in humans with ventilator-associated pneumonia decrease recurrent infection by resistant pathogens,[13] suggesting that decreasing selection pressure may preserve AMD efficacy.[14,15] Dogs with serious infections commonly are prescribed AMDs for extended periods[16] to limit recurrence or novel infection development,[17] but shorter AMD treatment durations in humans with serious infections do not worsen outcomes[13,18] and are recommended.[19] Evidence is accumulating in dogs that short AMD courses are safe and effective,[20,21] but determining the optimal time to discontinue AMDs is challenging.

In humans, biomarker measurements are used to guide AMD administration[22] and support decisions to discontinue treatment.[23-26] Decreases in biomarker concentrations below specific cutoffs, or decreases ≥80% from peak concentrations enable AMD discontinuation while minimizing the risk of relapse because of inadequate infection control.[27] For instance, decreases in procalcitonin (PCT) concentrations enable early AMD discontinuation in humans with secondary peritonitis without worsened outcomes.[25] Similarly, therapeutic decision-making algorithms incorporating C-reactive protein (CRP) for humans with sepsis (primarily caused by pneumonia and bacteremia) enable safe AMD discontinuation.[28,29] Studies of dogs with pyometra suggest that postoperative increases in CRP concentrations identify postoperative wound infections,[30] whereas CRP measurement may help shorten AMD treatment in dogs with pneumonia.[21] Observational studies of dogs with sepsis suggest that various biomarkers including PCT,[31-33] cell-free DNA (cfDNA),[34-36] high-mobility group box-1 (HMGB1),[37,38] and inflammatory cytokines,[39-41] warrant further investigation to determine which provide the best therapeutic guidance. To maximize the utility of these biomarkers as therapeutic guides, temporal patterns must be established in treated dogs with naturally-occurring disease.

Our overall objective was to investigate temporal changes in blood biomarker concentrations in dogs undergoing treatment for pulmonary and intra-abdominal infections. We aimed to describe clinicopathologic variables and inflammatory biomarker concentrations over time in dogs treated for pneumonia, septic peritonitis and pyometra, compare time to normalization of biomarker concentrations with the duration of clinician-directed AMD treatment, and contrast the time to biomarker normalization in dogs with distinct sources of infection. We hypothesized that inflammatory biomarker concentrations would decrease with treatment, normalize before clinicians discontinue AMDs and decrease earlier in dogs in which control of the source of infection can be surgically achieved.

2 | MATERIALS AND METHODS

2.1 | Study design

Ours was a prospective observational cohort study of client-owned dogs admitted to the Cornell University Hospital for Animals with pneumonia, septic peritonitis or pyometra. Pneumonia was diagnosed based on respiratory distress, cough or tachypnea (respiratory rate > 30 breaths/min or $PaCO_2$ <35 mm Hg), a cranioventrally distributed interstitial or alveolar pattern and a risk factor for aspiration pneumonia (eg, recent anesthesia or sedation, regurgitation or vomiting, laryngeal or pharyngeal dysfunction, esophageal or neurologic disease) or a risk factor for community-acquired pneumonia (eg, recent communal housing, exposure to a contagious respiratory pathogen, recent history of upper respiratory tract disease).[42] Septic peritonitis was diagnosed based on a positive bacterial culture of peritoneal fluid, presence of intracellular bacteria in peritoneal fluid cytology, documented perforation of the gastrointestinal tract, or radiographic evidence of free gas within the peritoneal cavity unrelated to a recent abdominal procedure.[32,43,44] Pyometra was diagnosed based on compatible history or clinical signs (eg, polyuria, polydipsia, vomiting, purulent vulvar discharge), diagnostic imaging findings indicating a distended fluid-filled uterus and a surgically-confirmed final diagnosis of pyometra. Dogs <5 kg were excluded to minimize risks associated with collection of additional blood samples for the study. Dogs were excluded if clients declined treatment recommendations or if the dogs were euthanized before initiation of treatment. Dogs were enrolled with written informed client consent. The local Institutional Animal Care and Use Committee approved the study (#2014-0053). The literature suggests that normalization of inflammatory biomarker concentrations can identify resolution of sepsis.[30,45-47] Normalization was defined as the point at which biomarker concentrations decreased to within the reference interval (RI) or decreased to 20% of their peak concentration.[23,26,48] The number of dogs necessary to distinguish peak biomarker concentrations from 20% peak results was estimated online (quantitativeskills.com/sisa/calculations/samsize.htm) using data on C-C motif chemokine ligand 2 (CCL2), HMGB1 and cfDNA concentrations from prior studies.[34,37] It was estimated that 27 dogs would be needed to detect a 1-way difference in biomarker concentrations with 80% power at $P < .05$. A 1-way difference was chosen because there was no biological rationale for an

increase in biomarker results with treatment. Estimates of mortality and loss to follow-up of 20% each were incorporated, and hence planned enrollment was 40 dogs.

2.2 | Case management and evaluation

Attending clinicians determined case management, including AMD type and duration of treatment. Study clinicopathologic and radiographic data were made available, but biomarker data were not disclosed. Signalment and physical examination findings at hospital admission were recorded. Blood gases and electrolyte and lactate concentrations were measured using point-of-care devices (RapidPoint 500, Siemens Healthcare, Malvern, PA; Lactate Pro, Arkray, Edina, MN). Cultures of blood, peritoneal fluid or airway samples were performed by a reference laboratory (Animal Health Diagnostic Center, Ithaca, NY). Venous blood gas analyses (RapidPoint 500, Siemens Healthcare) were performed immediately after sample collection. Complete blood counts (ADVIA 2120, Siemens Healthcare) with clinical pathologist review and serum biochemistry profiles (Cobas C501, Roche Diagnostics, Indianapolis, IN) were analyzed immediately whenever possible, and always within 48 hours of sample collection. Mentation score as well as blood glucose, albumin and lactate concentrations and platelet count were used to calculate illness severity scores ($APPLE_{fast}$).[49,50] Outcome at discharge was recorded as survived, died or euthanized. Blood samples were collected at study entry (D1), and then on days 3, 7, 14, 28 and 60, representing admission (D1) and recovery (D60) and typical AMD prescription durations. Blood samples were collected into evacuated tubes (Vacutainer, BD, Franklin Lakes, NJ) containing no additive (serum biochemistry and cytokine analyses), 3.2% sodium citrate (biomarker analyses), lithium heparin (APPs), or K_2-EDTA (CBCs).

2.3 | Inflammatory biomarkers

After sample collection, serum and plasma (heparin, citrate) were promptly prepared (within 5 minutes) from whole blood by centrifugation (1370 g, 10 minutes; Ultra-8V Centrifuge, LW Scientific, Lawrenceville, GA). Plasma was decanted into polypropylene tubes (Polypropylene Screw-Cap Microcentrifuge Tubes, VWR, Radnor, PA) with some plasma deliberately left behind to minimize cell contamination and then rapidly frozen (within 5 minutes) at $-80^{\circ}C$ pending batch analysis. Samples were shipped overnight on dry ice in 3 batches for singlet APP analyses (APP Laboratory, University of Miami, Miami, FL) using assays validated for dogs.[51-55] Maximal storage time before analysis was 12 months (median, 7). Plasma CRP concentrations were quantitated using an anti-human CRP reagent (Randox Laboratories, Kearneysville, WV) on a Daytona RX analyzer (Randox). Concentrations of SAA were quantitated using Vet-SAA (Eiken Chemical Co, Tokyo, Japan). Haptoglobin concentrations were quantitated using a phase colorimetric assay (Tri-DD, Boonton, NJ). Quality controls were performed and analyzers maintained according to manufacturer recommendations. The APP reference intervals (RIs) were CRP (0-20 μg/mL), SAA (0-10 μg/mL), and haptoglobin (0-2 mg/mL).

Citrate plasma concentrations of procalcitonin, HMGB-1 and nucleosomes were quantitated in duplicate using commercial ELISA kits (Canine procalcitonin, BioVendor, Asheville, NC; HMBG-1 ELISA, IBL-International, Morrisville, NC; Cell Death Detection ELISA-Plus, Roche, Indianapolis, IN) analyzed using a benchtop plate reader (Synergy H1 Hybrid; Gen-5, BioTek, Winooski, VT).[31,56-59] Plasma nucleosomes concentrations were scaled against pooled normal canine plasma (1.0 arbitrary units).[60] Reference intervals for procalcitonin were 9.4 to 93.3 pg/mL.[31] Citrate plasma cfDNA concentrations were measured in triplicate using a benchtop analyzer (Qubit 3.0 Fluorometer, Life Technologies, Carlsbad, CA) and relevant reagents (Quant-iT dsDNA HS reagent, Life Technologies).[34,35] Serum concentrations of CCL-2, C-X-C motif chemokine ligand-8 (CXCL8) and keratinocyte chemoattractant-like (KC-like) were quantitated in duplicate using a commercial multiplex assay (CCYTOMAG 90K, Milipore Sigma, Burlington, MA), with a benchtop flow cytometer (Luminex, BioRad, Hercules, CA).[40,61] Cytokine concentrations were calculated from standard curves generated from manufacturer standards. Sample concentrations reported by the analysis software were used as the quantitated concentrations. Where results were reported out-of-range high, the highest standard concentration was imputed; where results were reported out-of-range low, the manufacturer-stated minimal detectable concentrations were imputed: 21 pg/mL (CCL2), 21.7 pg/mL (CXCL8), 5.3 pg/mL (KC-like) to enable nonparametric statistical analyses. For all replicate biomarker measurements, mean results were used for subsequent analyses.

2.4 | Statistical methods

Data were assessed for normality using the D'Agostino Pearson test and appropriate descriptive statistics were calculated. Patient characteristics, physical examination findings and clinicopathologic results on D1 were compared among disease processes using mixed-effects models or the Kruskal-Wallis test and *P*-values adjusted using the Benjamini-Hochberg False Discovery Rate method ($Q = 5\%$).[62] Biomarker concentrations over time were compared by Kruskal-Wallis testing because all variables had some nonparametric data, and missing values precluded a repeated-measures test. Data from each time point were compared with D60 using Dunn's multiple comparisons tests. Scatterplots of biomarkers over time were inspected and geometric mean over time plotted for CRP and SAA as reported previously.[21] Additionally, CRP and SAA concentrations over time for each dog were plotted and time to normalization individually estimated, assuming biomarker concentrations changed linearly between observations. Time to normalization was compared with AMD duration by Kruskal-Wallis test with Dunn's post hoc correction. The Kruskal-Wallis test also was used to compare time to normalization of CRP and SAA among diseases. Duration of AMD administration in dogs with pneumonia was compared to dogs with septic peritonitis or pyometra using the Mann-Whitney *U* test. Correlations between biomarker concentrations were evaluated using Spearman's coefficients and scatterplots. Strength of correlation was assessed as follows: ≤0.4 weak, 0.41 to 0.5 mild, 0.51 to 0.6 moderate, 0.61 to 0.7 strong, 0.71 to 0.8 very strong, >0.8 excellent. Analyses

were performed using commercial software (Prism 9, GraphPad, La Jolla, CA) with alpha of .05.

3 | RESULTS

3.1 | Animals

Forty-two dogs were enrolled: 22 with pneumonia, 10 with septic peritonitis and 10 with pyometra. All dogs with septic peritonitis had surgically confirmed sources of infection. Seven dogs had visibly leaking intestine, 2 had intra-abdominal abscessation and 1 dog had bile peritonitis, cholelithiasis and positive peritoneal fluid bacterial cultures. Forty-one dogs survived to discharge and 1 dog with pneumonia was euthanized on D6 (disease severity). Two dogs were euthanized before D28 (underlying disease progression) with 3 dogs lost to follow-up, equivalent to a 28-day case fatality rate of 8% (3/39). Thirty-five dogs survived to D60, with 1 dog euthanized on D30 (disease recurrence). Population characteristics are summarized in Table 1, and comparisons among diseases identified significant between-group differences for age, sex distribution, and APPLE$_{fast}$ score (Table 1). The median (interquartile range [IQR]) duration of AMD treatment was 16 (12-23) days. A total of 132 AMDs were administered. Dogs were prescribed a median of 3 (3, 4) AMDs from 2 (2, 3) distinct AMD classes. The most prescribed AMDs were aminopenicillin/beta-lactamase inhibitor combinations (n = 75),

TABLE 1 Summary of population characteristics including complete blood count and serum biochemistry data from study entry

Parameter	All dogs (n = 42)	Pneumonia (n = 22)	Pyometra (n = 10)	Septic peritonitis (n = 10)	*P* value	$P_{Adjusted}$
Age (y)	6 (1-9.3)	2.5 (1-7)	10 (8.3-11.3)	3.5 (1-9)	.00	**.04**
Body weight (kg)	30.3 ± 16	30.3 ± 18.5	32.2 ± 9.4	28.6 ± 16.3	.88	.91
Sex (F/FS/M/MC)	13/13/7/9	2/8/6/6	10/0/0/0	1/5/1/3	<.0001	**<.01**
T (°F)	103 ± 1.6	102.9 ± 1.7	102.4 ± 1.4	101.9 ± 1.6	.28	.36
HR (bpm)	128 ± 29	117 ± 27	144 ± 27	135 ± 29	.03	.1
RR (bpm)	52 ± 22	59 ± 24	36 ± 14	53 ± 15	.04	.1
SAP (mm Hg)	138 ± 22	141 ± 18	145 ± 24	126 ± 25	.12	.22
MAP (mm Hg)	105 ± 18	109 ± 18	105 ± 14	97 ± 20	.23	.33
DAP (mm Hg)	84 ± 21	88 ± 23	81 ± 19	80 ± 20	.55	.66
SpO_2 (%)	96 (93-97)	94 (93-97)	98 (93-99)	98 (97-98)	.05	.12
APPLE$_{fast}$ score	20 (14-26)	15 (13-20)	22 (20-27)	25 (18-30)	.00	**<.05**
LoH (d)	3.8 (2.5-6.3)	3.5 (2-6)	3 (2.5-4.3)	6.5 (4.3-7.8)	.02	.09
AMD duration (d)	16 (12-23)	16 (12-24)	16 (10-18)	16.5 (12-23)	.67	.72
AMDs prescribed (n)	3 (3-4)	3 (3-4)	3 (3-3)	3 (3-4)	.6	.69
AMD classes (n)	2 (2-3)	2 (2-3)	2 (2-2)	2 (2-3)	.38	.47
Lactate	1.7 (1.3-2.6)	1.4 (1.2-2.1)	2.2 (1.7-2.6)	1.8 (1.3-4.1)	.17	.22
HCT (%)	43 ± 9.5	46 ± 7.4	38 ± 10.8	39 ± 10.5	.04	.1
Leukocytes (×10^3/μL)	15.3 (8.3-24.8)	12.1 (7.2-17.1)	23.9 (18.8-31.3)	22.6 (7.2-29.9)	.02	.08
Neutrophils (×10^3/μL)	11.3 (5.7-17.6)	9.0 (5.1-14)	17.3 (12.3-20.1)	14 (3.3-21.3)	.08	.16
Bands (×10^3/μL)	1.3 (0.1-3.3)	0.8 (0-1.6)	2.2 (0.3-7.4)	2.1 (1.2-6.9)	.06	.14
Lymphocytes (×10^3/μL)	1.2 (0.6-2.3)	0.9 (0.5-1.5)	2.7 (1.1-3.5)	1.4 (0.8-2.4)	.01	.08
Monocytes (×10^3/μL)	0.9 (0.4-2.3)	0.7 (0.3-1.5)	2.7 (1.4-4.7)	0.6 (0.2-1.7)	.02	.08
Eosinophils (×10^3/μL)	0 (0-0.2)	0 (0-0.2)	0 (0-0.3)	0 (0-0.3)	.97	.97
Platelets (×10^3/μL)	223 (143-304)	223 (154-296)	247 (162-490)	185 (127-319)	.65	.72
Albumin (mg/dL)	2.9 ± 0.7	3.2 ± 0.7	2.7 ± 0.5	2.6 ± 0.8	.08	.17
ALT (U/L)	53 (29-89)	55 (43-83)	29 (19-62)	72 (28-352)	.23	.33
Total bilirubin (mg/dL)	0.1 (0.1-0.2)	0.1 (0-0.1)	0.1 (0.1-0.3)	0.1 (0.1-0.5)	.24	.33
BUN (mg/dL)	10 (8-17)	10 (8-15)	9 (9-12)	17 (9-30)	.19	.32
Cholesterol (mg/dL)	246 (180-313)	238 (168-265)	321 (241-374)	215 (125-341)	.03	.1
Creatinine (mg/dL)	0.7 (0.6-0.9)	0.7 (0.6-0.8)	0.7 (0.6-0.8)	1.0 (0.7-1.1)	.24	.33

Note: Data are presented as mean ± SD for normally distributed data and median (IQR) for nonnormally distributed data. Comparisons between data from dogs with different disease processes were compared with Kruskal-Wallis tests or χ^2. Raw *P* values and those following adjustment for multiple comparisons using the Benjamini-Hochberg false discovery rate method ($Q = 5\%$) are presented. *P* values displayed in bold font remained significant at $P < .05$ after correction for multiple comparisons.

specifically ampicillin/sulbactam (n = 39) and amoxicillin/clavulanate (n = 36) and fluoroquinolones (n = 38; enrofloxacin [n = 37], pradofloxacin [n = 1]). Other AMD classes included first-generation cephalosporins (n = 4), third-generation cephalosporins (n = 4), carbapenems, lincosamides, nitroimidazoles, tetracyclines (all n = 2) and aminopencillins, amphenicols and potentiated sulfonamides (all n = 1).

3.2 | Complete blood counts and biochemistry panels

Serial CBCs identified numerous differences between D60 results and those at earlier time points (Figure S1). Temporal patterns were apparent for all variables, except lymphocyte counts, with most D60 results being within local RIs and significantly different from at least 1 earlier time point. For leukocyte and neutrophil counts, numerical increases in median counts were found between D1 and D3 with both results higher than the upper RI bound. Band neutrophil counts were highest on D1, with counts on D1 and D3 significantly higher than on D60; median counts were within the RI on D7. On serum biochemistry panels, significant increases in the concentrations of urea, albumin, and creatinine between early time points and D60 were observed (Figure S2). For albumin, these changes typically represented increases from subnormal results before D7 to normal on D60. In contrast, for blood urea nitrogen and serum creatinine concentrations, these changes represented significant increases in concentration, but most concentrations remained within RIs throughout. Five dogs had increases in serum creatinine concentration of 200% to 299% (veterinary acute kidney injury [VAKI] stage 2) and 1 dog had an increase of ≥300% (VAKI stage 3). Two dogs had serum creatinine concentrations above the RI at D60 (Data S1).[63]

3.3 | Acute phase proteins and procalcitonin

Concentrations of CRP and PCT were maximal on D1, SAA concentrations were maximal on D3, and haptoglobin concentrations were

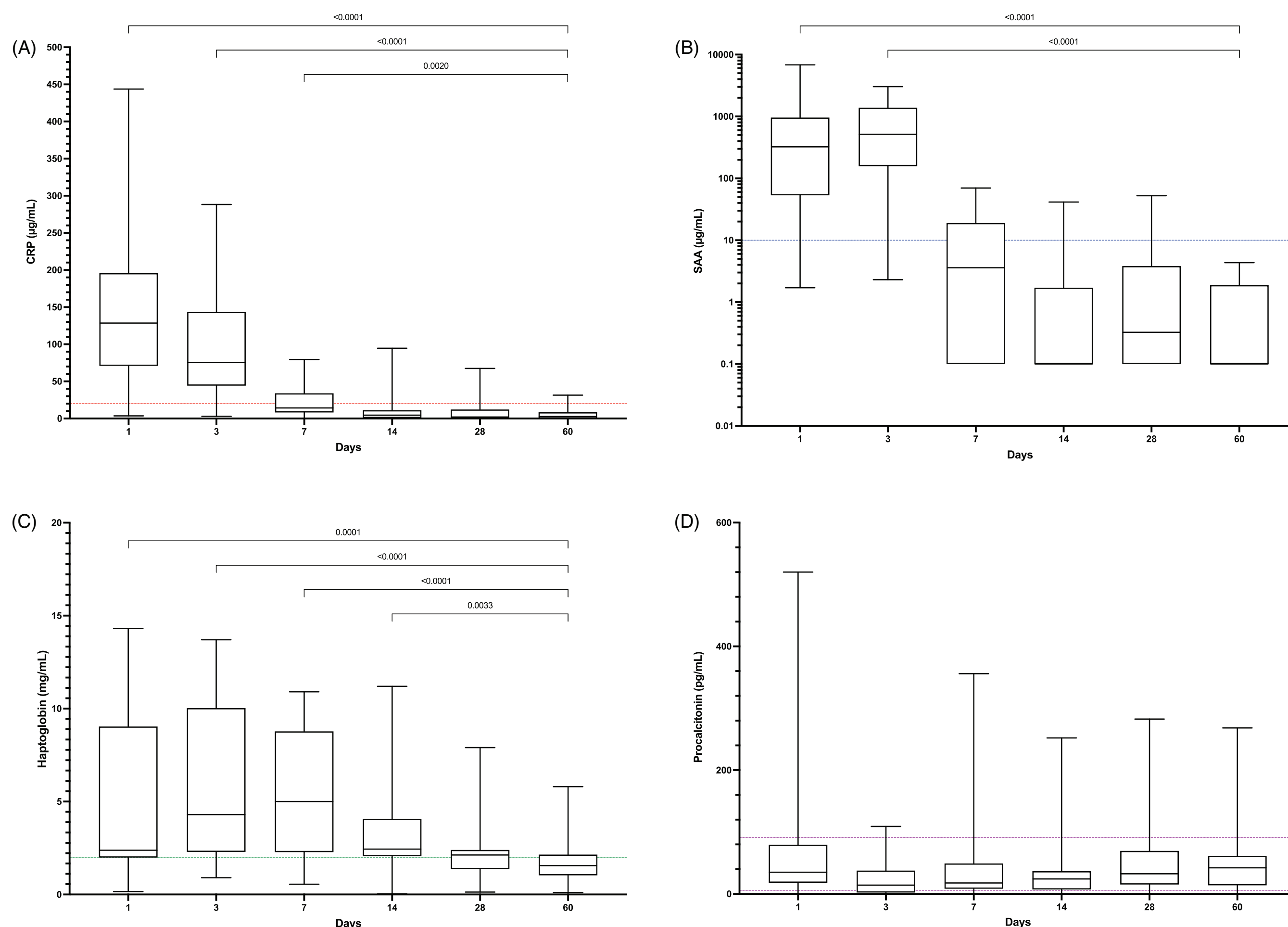

FIGURE 1 (A-D) Box-whisker plots of C-reactive protein (CRP), serum amyloid A (SAA), haptoglobin, and procalcitonin (PCT) concentrations over time in dogs with severe bacterial infections treated with standard care including antimicrobial drugs and source control where applicable. Horizontal solid lines represent medians, boxes represent the interquartile range (25%-75%), and whiskers represent minimal and maximal results. Horizontal dotted lines represent the relevant reference intervals. Only differences between concentrations at D60 and earlier time points that were significant (*P* < .05) by Kruskal-Wallis with Dunn's post hoc multiple comparisons tests are represented

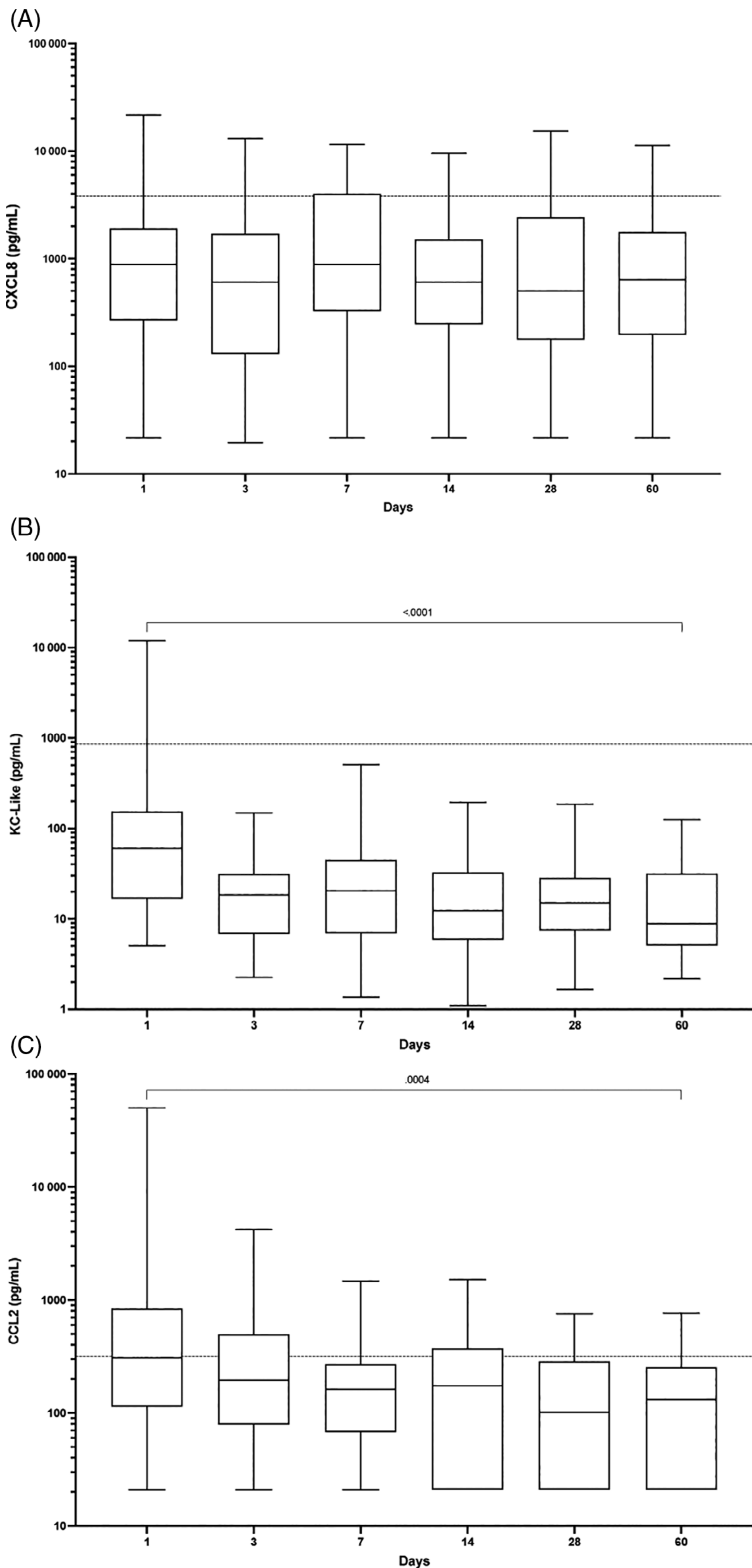

FIGURE 2 (A-C) Box-whisker plots of C-C motif chemokine ligand 2 (CCL2), C-X-C motif chemokine ligand 8 (CXCL8), and keratinocyte chemoattractant-like (KC-Like) concentrations over time in dogs with severe bacterial infections treated with standard care including antimicrobial drugs and source control where applicable. Horizontal solid lines represent medians, boxes represent the interquartile range (25%-75%), and whiskers represent minimal and maximal results. Concentrations are represented on a $\log_{10}$ scale (Y-axes). Only differences between concentrations at D60 and earlier time points that were significant (*P* < .05) by Kruskal-Wallis with Dunn's post hoc multiple comparisons tests are represented

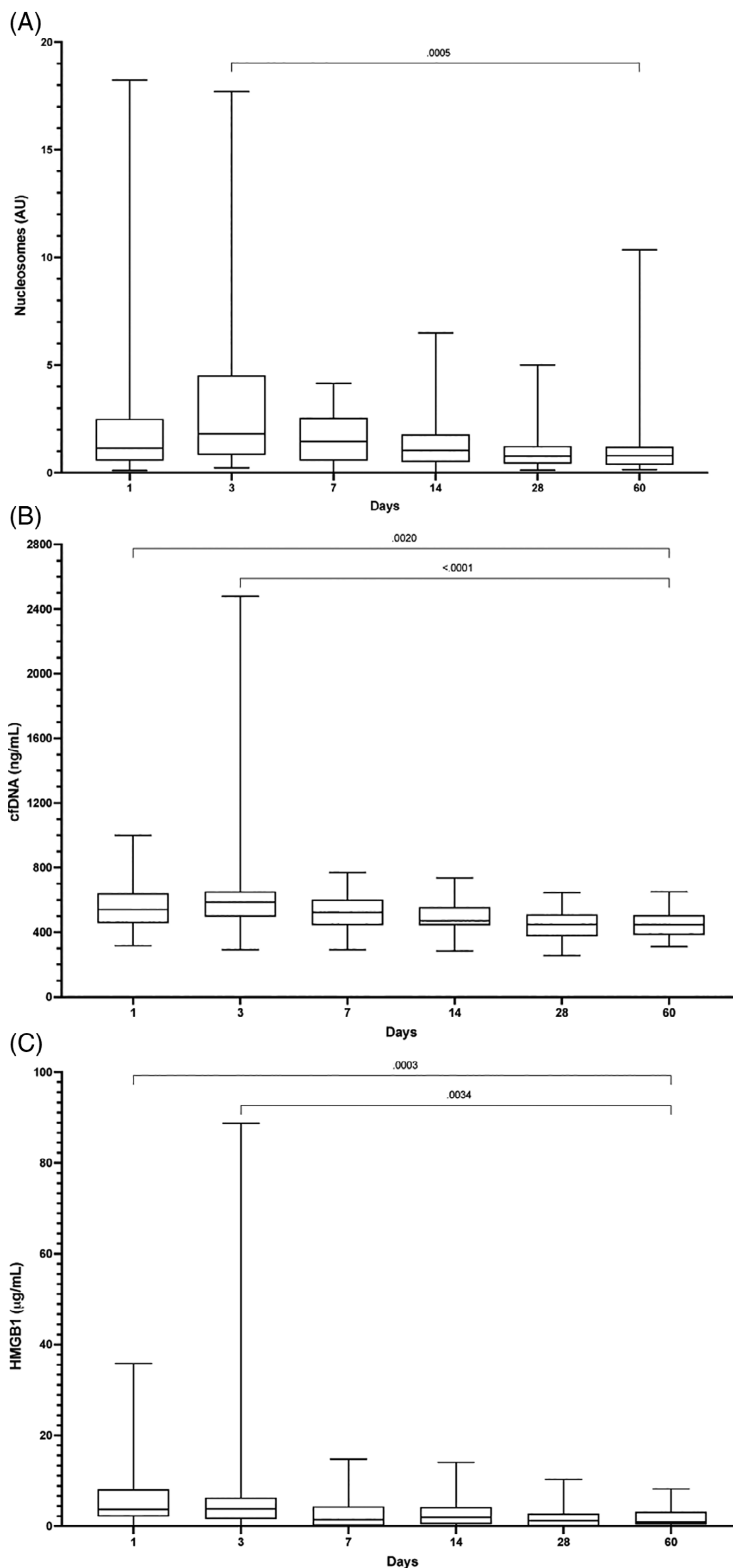

FIGURE 3 (A-C) Box-whisker plots of nucleosome, cell-free DNA (cfDNA), and high mobility group box-1 (HMGB1) concentrations over time in dogs with severe bacterial infections treated with standard care including antimicrobial drugs and source control where applicable. Horizontal solid lines represent medians, boxes represent the interquartile range (25%-75%), and whiskers represent minimal and maximal results. Horizontal dotted lines represent the relevant reference intervals. Nucleosome concentrations are represented in arbitrary units (AU), scaled against pooled normal canine plasma given an AU value of 1.0. Only differences between concentrations at D60 and earlier time points that were significant ($P < .05$) by Kruskal-Wallis with Dunn's post hoc multiple comparisons tests are represented

maximal on D7. Concentrations of APPs and PCT followed distinct temporal patterns (Figure 1). Concentrations of CRP decreased in curvilinear fashion described by a 1-phase exponential decay function and by D14 were not significantly different from D60 (Figure S3). Concentrations of SAA were significantly increased on D1 and D3 relative to D60, and then decreased rapidly. Median haptoglobin

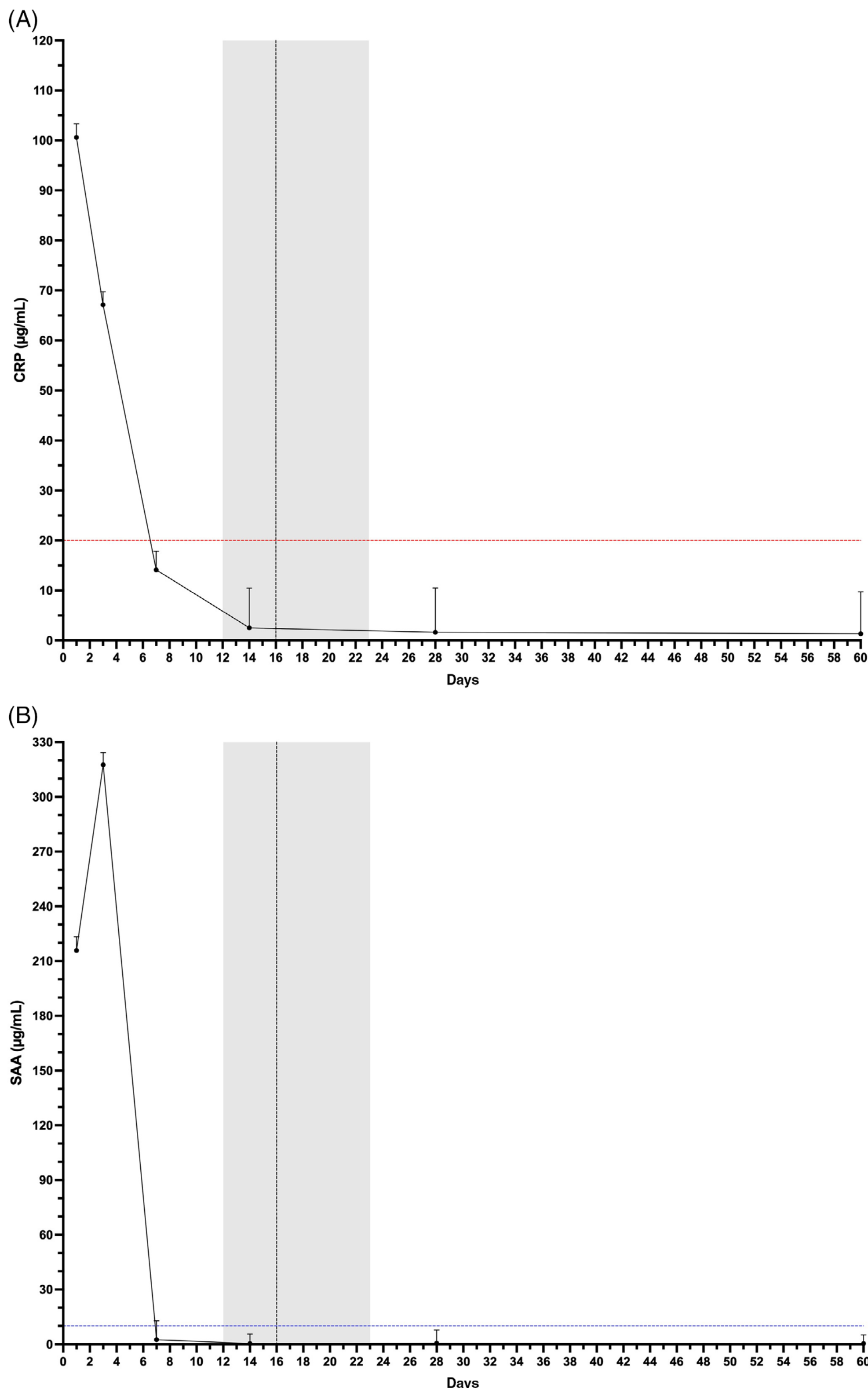

FIGURE 4 (A and B) Line plots of the geometric mean concentrations of C-reactive protein (CRP) and serum amyloid A (SAA) over time in dogs with severe bacterial infections treated with standard care including antimicrobial drugs and source control where applicable. Dots represent geometric means of biomarker concentrations with geometric SD vertical error bars (1-tail only displayed for clarity). The biomarker concentrations were assumed to change linearly between time points. The separate horizontal lines (green) represent the median (dot) with interquartile range (whiskers) of the duration of antimicrobial drug (AMD) prescribing in the cohort of dogs. Horizontal dotted lines represent the relevant reference intervals

concentrations increased from D1 to D7 and then decreased. Haptoglobin concentrations on D1, D3, D7, and D14 were significantly higher than on D60. Concentrations of PCT were not significantly different from D60 at any previous time and most were within the RI. Temporal patterns of APP concentrations by subgroup were consistent with those of the entire population (Figure S5).

3.4 | Cytokines

Concentrations of CXCL8 at each time varied considerably by approximately 1000-fold between the lowest and highest measured concentrations, but no differences between D60 and any previous time were observed. Concentrations of KC-Like and CCL2 also varied substantially.

For CCL2, 21% of samples had values below the limit of quantitation, particularly at later time points. For KC-Like and CCL2, concentrations were maximal on D1 and were significantly different from D60 (Figure 2). No other differences between D60 and earlier times were observed. The temporal patterns of cytokine concentrations by subgroup were consistent with those of the entire population (Figure S6).

3.5 | Nucleosomes, cfDNA, and HMGB1

Temporal patterns of biomarkers putatively associated with neutrophil extracellular trap formation (NETosis) were similar, with highest concentrations occurring on D3. The D3 concentrations were significantly higher than on D60 for all 3 markers (Figure 3). Concentrations of cfDNA and HMGB1 also were significantly higher on D1 than on D60. For cfDNA and HMGB1, the extent of variation decreased over time, but all concentrations overlapped substantially between times.

3.6 | Bivariate biomarker correlations

Bivariate analyses identified 28 significant correlations. Of these, 24 were weak ($r_s \le 0.4$) and were not further evaluated. Mild positive correlations were found between KC-Like and CCL2 (r_s 0.432, $P < .0001$) and CXCL8 and KC-Like (r_s 0.465, $P < .0001$), a moderate positive correlation was found between cfDNA and CRP (r_s 0.516, $P < .0001$) and a very strong positive correlation was found between CRP and SAA (r_s 0.757, $P < .0001$, Figure S4). For further assessment of the CRP and SAA correlation, respective RIs were overlaid on the scatterplot to determine classification of individual results by each biomarker. The 2 biomarkers agreed on classification as normal (n = 114) or abnormal (n = 85) for 88.4% of paired samples, with 11.6% of samples classified differently by the 2 biomarkers, more commonly abnormal CRP and normal SAA (n = 19) than normal CRP and abnormal SAA (n = 7, Figure S4).

3.7 | Biomarkers vs antimicrobial drug prescribing

Geometric mean CRP and SAA concentrations plotted over time suggest these biomarkers normalized at approximately D7 (Figure 4). Normalization of CRP and SAA concentrations occurred significantly earlier than the time at which AMDs were discontinued (Figure 5). For CRP, the median (IQR) times to 20% peak and to RI were 7 (6-9) and 7 (6-12) days, respectively, and for SAA, the median (IQR) times to 20% peak and to RI were 4 (4, 5) and 6 (5-8) days, respectively; compared to a median (IQR) AMD duration of 16 (12-23) days. No differences were observed in AMD prescribing duration among disease

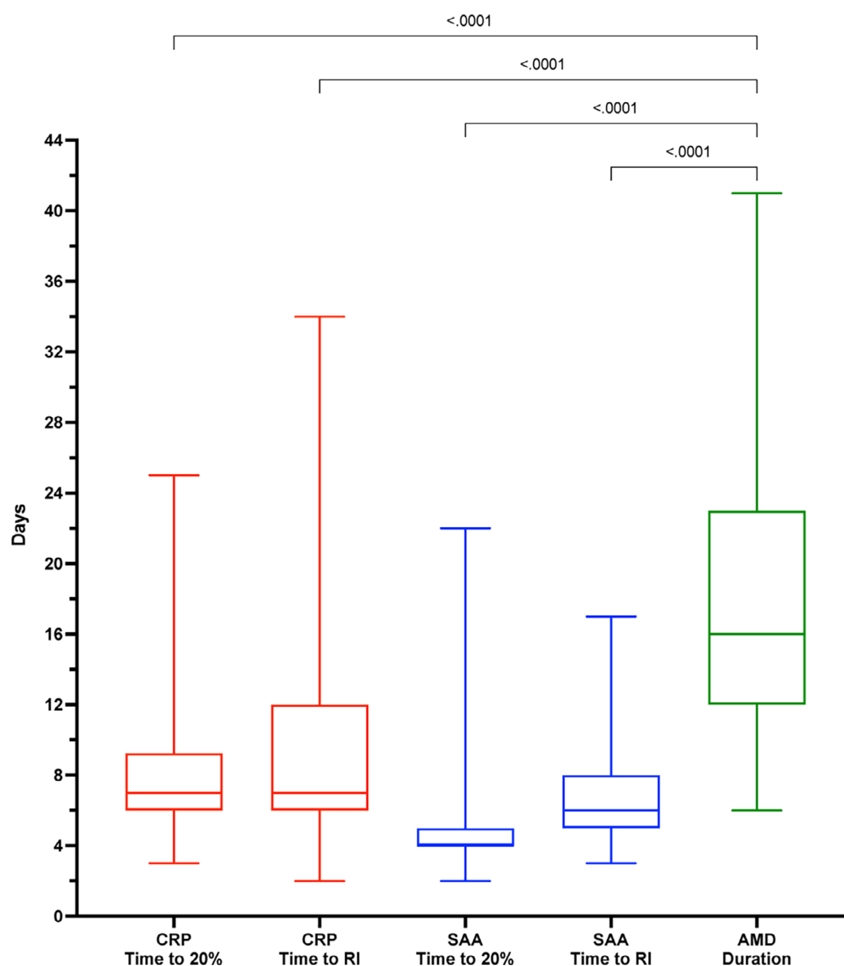

FIGURE 5 Box-whisker plots comparing time (days) for concentrations of C-reactive protein (CRP) (red) and serum amyloid A (SAA) (blue) to decrease to 20% of peak concentrations and to within the reference interval (RI) with the duration of antimicrobial drug (AMD) treatment (green). Horizontal solid lines represent medians, boxes represent the interquartile range (25%-75%), and whiskers represent minimal and maximal results. All differences between time to biomarker normalization and duration of AMD treatment significant ($P < .05$) by Kruskal-Wallis with Dunn's post hoc multiple comparisons tests are represented

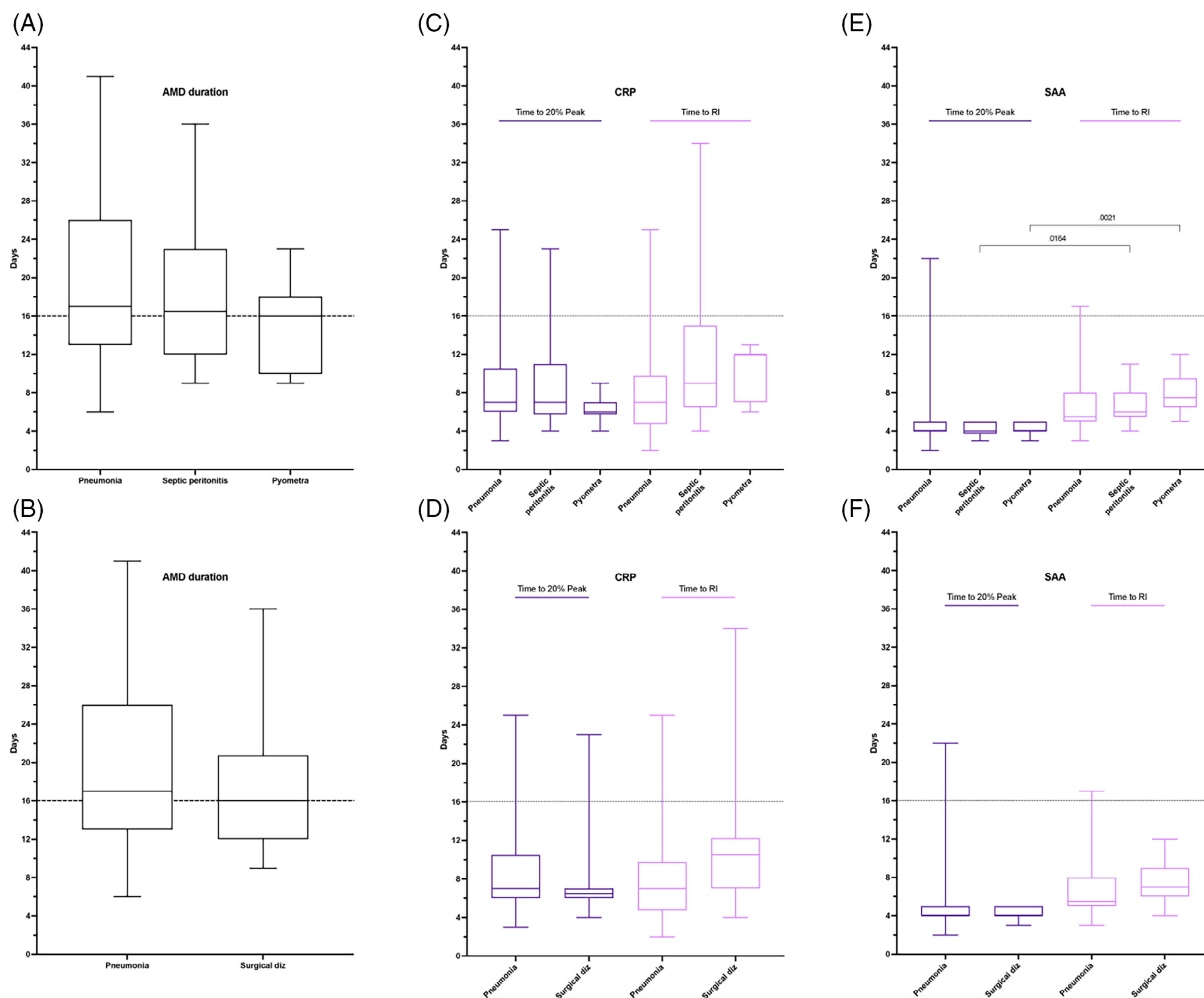

FIGURE 6 Box-whisker plots comparing the duration of antimicrobial drug (AMD) prescribing across the 3 different disease processes (A) and between pneumonia and diseases treated by surgical source control (septic peritonitis and pyometra) (B). Also displayed are box-whisker plots comparing the time to normalization (time to 20% of peak concentrations and time to within the reference interval [RI]) for CRP (C and D) and SAA (E and F) across the three different disease processes and between pneumonia and diseases treated by surgical source control (septic peritonitis and pyometra). Only differences between concentrations at D60 and earlier time points that were significant ($P < .05$) by Kruskal-Wallis with Dunn's post hoc multiple comparisons tests are represented

processes, or between diseases managed medically (pneumonia) and those managed surgically, or in time to CRP or SAA normalization between pneumonia and surgical conditions (Figure 6).

4 | DISCUSSION

Our objective was to investigate temporal changes in blood biomarker concentrations in dogs undergoing treatment for pulmonary and intra-abdominal infections. In general, inflammatory biomarker concentrations were highest on D1 or D3 and decreased over time, with normalization of biomarker concentrations typically by D14 except for haptoglobin. Previously recognized temporal responses of APPs and other biomarkers to an inflammatory stimulus were recapitulated here.[64-68] Haptoglobin is a minor APP in dogs with delayed onset and resolution relative to CRP and SAA,[65,69] and its delayed normalization here was consistent with a study of dogs with pyometra.[30]

We also aimed to compare time to biomarker normalization with AMD duration and the time to biomarker normalization across diseases. Concentrations of CRP and SAA decreased to 20% peak and to within RIs significantly earlier than clinicians discontinued AMDs in all 3 groups of dogs. For CRP, normalization occurred on average 9 days before AMDs were discontinued, whereas for SAA, normalization occurred on average 10 to 12 days before AMDs were discontinued based on return to the RI and on time to 20% peak, respectively. These observations were consistent with a report of dogs with

pneumonia,[21] suggesting that CRP and SAA measurement can aid clinicians in therapeutic decision-making. We did not however control AMD prescribing duration in our study. The temporal patterns of CRP and SAA concentrations in our study were similar to those reported for dogs with bacterial pneumonia.[21] These findings may be generalizable to dogs with other types of infection, and additional studies to confirm this hypothesis are warranted. Additionally, randomized controlled trials comparing APP-guided AMD prescribing with standard clinician-directed care are warranted to determine if our observational data can be translated into clinical practice.

We hypothesized that biomarker concentrations would decrease earlier in dogs in which source control could be surgically achieved compared with pneumonia that was managed medically. Our data do not support this hypothesis, because no differences in time to normalization of CRP or SAA were observed among disease processes, or between medically versus surgically managed infections. Dogs with pneumonia had lower illness severity scores than did dogs with septic peritonitis or pyometra, which could have masked a difference in the time to biomarker concentration normalization. Surgical source control is essential for management of pyometra or septic peritonitis, but it may not be sufficient such that AMDs also are needed to help control any residual or disseminated infection not amenable to surgical intervention. We did not however incorporate a control group of animals that were not treated with antimicrobials. The animals in our study were client-owned, and it would not have been ethical to withhold AMDs from a group of them. As such, we do not know what the time course of biomarkers such as CRP or SAA would be in patients managed without AMDs, for instance dogs with pyometra treated with surgery alone.

Decreasing AMD treatment duration should limit selection pressure,[14,15] and in humans shorter AMD duration decreases AMR development.[13,70,71] Dogs are commonly prescribed AMDs for several weeks, ostensibly to limit recurrence,[17] but humans with intra-abdominal infections are treated effectively with only 4 days of AMDs.[18,72] In a study of dogs with bacterial pneumonia,[21] normalization of CRP was used to guide AMD use with treatment discontinued 5 to 7 days after CRP was <25 μg/mL. This approach significantly shortened AMD duration without negative consequences. It is uncertain how that protocol would have affected our study. Using CRP normalization and an additional 5 to 7 days would have resulted in AMD administration for 12 to 14 days, fewer than the 16 day median observed in our study. Use of SAA normalization would have resulted in 11 to 13 days of AMDs, again a shorter duration. An important but unanswered question is whether AMDs could be safely discontinued when CRP and SAA normalized, rather than continuing AMDs for an additional 5 to 7 days. The human medical literature suggests doing so would be safe,[27-29,73] but prospective trials will be needed to confirm this possibility in dogs. It is uncertain if CBC results could substitute for biomarkers to guide early AMD discontinuation. Our study suggests not, because D14 neutrophil counts were significantly increased relative to D60, and some dogs had band neutrophilia on D14. Clinicians managing dogs in our study did not have access to biomarker concentrations to guide decision-making, and likely relied upon CBC results to assess inflammation. This aspect of study design may explain the 16-day median AMD duration we observed. We did not compare the specificity and sensitivity of APP measurements with CBC variables for AMD decision-making, but there are many other causes for neutrophilia and left shift in critically ill dogs in addition to ongoing infection.

Dogs in our study received a median of 3 AMDs. This result is most likely because of frequent initial, empirical use of parenteral ampicillin/sulbactam and enrofloxacin, followed by usage of PO amoxicillin/clavulanate to facilitate hospital discharge. Parenteral amoxicillin/clavulanate is not available at our institution and hence we recorded this approach as usage of 3 drugs. Some dogs received >3 AMDs for various reasons including relapse, recurrence or suspected treatment failure, poor client compliance resulting in use of additional medications, use of AMDs for concurrent but potentially related infections (eg, postoperative skin infection), and cases in which AMDs were used for unrelated infections that would have contributed to antibiosis for the primary disease.

We did not assess the utility of biomarkers for identification of relapse or reinfection, but the SAA results suggest this outcome may have occurred in a few dogs between D14 and D60, and this might explain the extended AMD durations in some dogs (eg, a puppy with pneumonia prescribed AMDs for 41 days). A prior study of pyometra in dogs suggested new increases in CRP concentration could help identify postoperative wound infections,[30] which may also occur after stifle surgery.[53] Post hoc evaluation of SAA data identified 3 cases with concentrations >20 μg/mL on D28. One was from a dog with septic peritonitis euthanized for disease recurrence on D30 and 1 was from a dog with pneumonia that was still receiving veterinary care for unresolved disease. Medical record review did not identify an explanation for the high D28 SAA concentration in the third dog, but this animal was frequently in communal housing at the time and hence exposure to community-acquired respiratory pathogens is possible.

Other studies have assessed the prognostic value of CRP measurements. In dogs with CRP concentrations >100 μg/mL, a single CRP measurement was not prognostic,[74] but serial CRP measurements may be more useful. In dogs with sepsis, the decrease in CRP concentrations over the first 48 hours of hospitalization was significantly larger than in nonsurvivors.[47] We did not assess the prognostic value of biomarkers here because the low overall case fatality rates precluded such analyses. The overall illness severity in our study was lower than in other studies of dogs with sepsis,[33,34,50,75-77] which likely contributed to the higher survival rates in our study.

The strong correlation observed between CRP and SAA concentrations is consistent with a study of 500 dogs with systemic inflammation.[78] In that study, CRP and SAA agreed in 90% of the dogs and more dogs had abnormal CRP concentrations and normal SAA concentrations than had normal CRP and abnormal SAA concentrations. The same pattern was observed in our study, where agreement was found in 88.4% of samples, and the comparable scatterplot in the previous study[78] was very similar to that in our study. These findings suggest that CRP and SAA measurements in distinct populations of dogs with systemic inflammation are very reproducible and that CRP may be slightly more sensitive or have a higher false positive rate than SAA.

We measured PCT concentrations in addition to conventional APPs because PCT likely behaves comparably to other APPs in both humans and dogs.[79-81] In humans, PCT is an important diagnostic and prognostic biomarker in sepsis,[82] and a useful therapeutic guide for AMD initiation and discontinuation.[23,73,83,84] Previous studies in dogs suggest that PCT concentrations are increased in dogs with sepsis and nonseptic inflammation,[31,32] and predict organ dysfunction and outcome.[33] The range of PCT concentrations in our study was comparable to that of previous reports, but median concentrations were generally lower, and potentially attributable to lower illness severity in our cohort, where the median $APPLE_{fast}$ score was 20, compared with 22 and 24 in previous studies.[32,33] Lower illness severity in our study might have decreased the utility of PCT for identifying infection resolution by decreasing the magnitude of any changes from peak.

Local RIs for cytokines are not established at our institution, but others have suggested RIs for these variables in dogs specifically CXCL8 (0-3775 pg/mL), KC-Like (0-855 pg/mL), and CCL2 (0-317 pg/mL).[40] In our study, most CXCL8 and KC-Like concentrations were within these RIs. In contrast, for CCL2, 28.7% of all samples and 46% of samples on D1 had concentrations above the RI. Most CCL2 results decreased below the RI by D7 (Figure 2), suggesting that CCL2 might best identify resolution of inflammation. Other studies suggest that CCL2 is useful for assessing dogs with sepsis,[32,37,40,85] and with other causes of severe inflammation including trauma,[85,86] immune-mediated hemolytic anemia (IMHA),[40,61,85] pancreatitis,[87] cancer,[88-90] and babesiosis.[91-93] The positive correlations observed between pairs of cytokines suggest similar stimuli are responsible for the increased concentrations of these various molecules. This conclusion is corroborated by a study that found similar cytokine concentrations in dogs with IMHA and those with sepsis, indicating convergent cytokine responses in inflammatory diseases with a distinct pathogenesis.[40]

The concentrations of cfDNA, nucleosomes and HMGB1 were measured as putative NETosis biomarkers, although none is ideal for quantifying NETosis in dogs.[94] Previous studies of dogs with sepsis and systemic inflammation suggest these markers are not discriminatory for sepsis, perhaps because NETosis contributes to disease pathogenesis in IMHA,[95] gastric dilatation-volvulus (GDV) syndrome,[96] cancer,[97,98] trauma,[86,99] and pancreatitis.[100] Alternatively, these markers may be nonspecific for NETosis in dogs through release by necrotic and apoptotic cells.[94] In our study, these biomarkers all were increased on D3 relative to D60, with cfDNA and HMGB1 concentrations also increased on D1. Presently, RIs are not defined for these biomarkers in dogs such that time to 20% peak would be necessary to determine normalization. Of the 3 markers, cfDNA is most clinically applicable because it can be measured using point-of-care instruments,[34] whereas the other markers require ELISA assays with long turnaround times. Concentrations of the NETosis markers varied and overlapped considerably over time, limiting their usefulness for discriminating disease resolution. Prior studies in dogs with sepsis suggest their maximal utility is in the most severely affected animals. For instance, in dogs with sepsis, cfDNA concentrations predict bacteremia and mortality (when compared by ratio to neutrophil count),[34] whereas in GDV syndrome HMGB1 is associated with severity of tissue injury.[96] The comparatively low overall illness severity in our study may have blunted their utility.

Our study had some limitations. As indicated by $APPLE_{fast}$ scores and overall case fatality rates, our cohort had lower illness severity than other populations, potentially related to our eligibility criteria and possible selection bias by attending clinicians and clients. For severely ill animals, clinicians might have been reluctant to offer study enrollment, or clients reluctant to consent. We could not track how many cases were considered for enrollment but not enrolled, or how often consent was declined. As such, case selection bias is unquantifiable but may have occurred and could limit generalizability of our results. Illness severity should be accounted for in future randomized trials, through stratification or subgroup definition.

The time points in our study were designed to capture biomarker data for typical AMD prescription durations, but time intervals between sampling points were not constant and for cost, logistical and client compliance reasons additional sampling was not feasible. Important changes in biomarker concentrations that could have influenced interpretation might have been missed and we might not have identified the precise time biomarkers normalized. In estimating the time at which CRP and SAA concentrations normalized, we assumed linear changes in concentrations between time points. This assumption was necessary because we could not fit curves to data from every dog. To address this issue, we fitted a curve to the geometric mean concentration for CRP as previously described[21] (Figure S3). Reassuringly, this curve fit produced the same estimate of time to RI (7 days) as the median of normalization times adjudicated for each dog individually.

Our study population included dogs with pneumonia, septic peritonitis and pyometra and is inherently heterogeneous because of distinct pathogenesis and dissimilar infecting organisms, pathogen load and virulence. As such, our conclusions may not apply to every dog with these diseases and extrapolation of our results to dogs with other infections (eg, pyothorax, endocarditis, prostatitis) should be made cautiously. In addition, we cannot be certain that all dogs in our study had a bacterial infection or that infections were not polymicrobial. Specifically, some dogs presumptively diagnosed with aspiration pneumonia may have had pneumonitis without infection, [101,102] whereas the pathogens involved in dogs with community-acquired pneumonia may have included respiratory viruses and *Mycoplasma* spp. in addition to other bacteria.[103] All dogs in our study with septic peritonitis had visual confirmation of intestinal leakage or laboratory culture confirmation of bacterial infection, and some may have had concurrent fungal peritonitis that was not identified, but that contributed to inflammation and temporal changes in biomarker concentrations.[104,105] All biomarker assays that we employed either have been validated specifically in dogs or have been used frequently in dogs. However, few of the biomarker assays employed are routinely available diagnostic tests and many of the assay kits are intended for research use only. The most discriminant biomarkers (CRP and SAA) are widely available and can be quantified using high-throughput wet chemistry analyzers in reference laboratories, but appropriate caution should be employed when using data from our study to guide management of individual client-owned dogs in clinical settings.

Concentrations of CRP, SAA and haptoglobin were measured only in singlet, because the automated assays used for these biomarkers have the requisite precision, but this feature would make detection of an erroneously high or low or discordant value more difficult.

In summary, our study provides data on the temporal patterns of APPs, inflammatory cytokines, and NETosis markers in dogs with naturally-occurring pulmonary and intra-abdominal infections treated conventionally. These data could be useful for clinicians using biomarkers to inform patient management and for designing clinical trials. Our study found that CRP and SAA concentrations typically normalized within 7 days of initiating treatment, significantly earlier than clinicians discontinued AMDs. Our study suggests that serial biomarker measurements, in particular CRP and SAA, can help identify disease resolution and enable veterinarians to discontinue AMDs sooner without risking patient safety. Randomized controlled trials comparing APP-guided AMD prescribing with standard clinician-directed care are warranted and could enable early and safe AMD discontinuation, decrease client costs and risk of adverse drug reactions,[106] and minimize development of bacterial AMR.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Pradofloxacin was used off label in 1 dog. This drug does not have Food and Drug Administration approval in the United States for use in dogs but does have European Medicines Agency approval for use in dogs in Europe. Imipenem and meropenem were used in 1 dog.

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

Approved by the Cornell University IACUC, Protocol #2014-0053.

HUMAN ETHICS APPROVAL DECLARATION

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

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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