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Acute phase protein response and changes in lipoprotein particle size in dogs with systemic inflammatory response syndrome

Erica Behling-Kelly[1] | Carol E. Haak[1] | Patrick Carney[2] | Jessica Waffle[1] | Kelly Eaton[1] | Robert Goggs[2]

[1]Department of Population Medicine and Diagnostic Sciences, Cornell University, Ithaca, New York, USA

[2]Department of Clinical Sciences, Cornell University, Ithaca, New York, USA

Correspondence
Erica Behling-Kelly, Cornell University College of Veterinary Medicine, Clinical Pathology Laboratory, S1-062 Schurman Hall, 602 Tower Road, Ithaca, NY 14853, USA.
Email: eb58@cornell.edu

Abstract

Background: Improved methodology to measure acute phase proteins and determination of lipoprotein particle-size distribution (PSD) could be clinically useful in dogs with systemic inflammatory processes.

Objectives: Evaluate an immunoturbidometric assay for serum amyloid A (SAA) and lipoprotein PSD in dogs with sepsis, nonseptic systemic inflammation, and in healthy controls. Correlate dyslipidemic changes with SAA and C-reactive protein (CRP) concentrations.

Animals: Twenty-five dogs with sepsis, 15 dogs with nonseptic systemic inflammation, and 22 healthy controls.

Methods: Prospective, case-control study. Variables included SAA, CRP, and electrophoretic subfractionation of high- and low-density lipoproteins (HDL, LDL). Continuous variables were compared using ANOVA or Kruskal-Wallis tests with linear regression or Spearman's rank correlation used to assess relationships between variables.

Results: Median SAA and CRP concentrations were greater in dogs with sepsis (SAA 460 mg/L, interquartile range [IQR] 886 mg/L; CRP 133.2 mg/L, IQR 91.6 mg/L) and nonseptic inflammation (SAA 201 mg/L, IQR 436 mg/L; CRP 91.1 mg/L, IQR 88.6 mg/L) compared to healthy dogs (SAA 0.0 mg/L, IQR 0.0 mg/L; CRP 4.9 mg/L, IQR 0.0 mg/L) $P < .0001$. A cutoff of >677.5 mg/L SAA was 43.2% sensitive and 92.3% specific for sepsis. Low-density lipoprotein was higher in dogs with sepsis 29.6%, (mean, SD 14.6) compared to 14.4% (mean, SD 5.6) of all lipoproteins in healthy controls ($P = .005$). High-density lipoprotein was not associated with CRP but was negatively correlated with SAA (r_s −0.47, $P < .0001$). Subfractions of LDL and HDL differed between groups (all $P < .05$).

Abbreviations: APP, acute phase proteins; AUC, area under the curve; CI, confidence interval; CRP, C-reactive protein; HDL, high-density lipoprotein
LDL, low-density lipoprotein; NSI, nonseptic inflammation; PON-1, paraoxonase-1; PSD, particle-size distribution; SAA, serum amyloid A; SI, septic inflammation; TG, triglycerides; VLDL, very low-density lipoprotein.

Conclusions and Clinical Importance: Measurement of SAA using the immunoturbidometric assay evaluated in this study and lipoprotein PSD in dogs with inflammation might help distinguish septic from nonseptic causes of inflammation.

KEYWORDS
canine, CRP, lipid, SAA, sepsis

1 | INTRODUCTION

Accurate and timely identification and monitoring of systemic inflammation enables clinical decision-making. The positive acute phase proteins (APP), C-reactive protein (CRP), and serum amyloid A (SAA) are useful serum biomarkers of inflammation in dogs.[1] Serum lipoproteins transport some APP and have direct immunomodulatory functions in some species.[2,3] High-density lipoproteins (HDL) scavenge free cholesterol and lipid breakdown products and neutralize bacterial lipopolysaccharide.[4,5] Low plasma concentrations of HDL predict death in humans with pneumonia,[6,7] whereas HDL offers a protective benefit in mouse models of allergic airway inflammation.[8] The antioxidant functions of HDL are partially attributed to bound paraoxonase-1 (PON-1), which can be displaced by SAA.[9,10] PON-1 activity is lower in dogs with sepsis as compared to dogs with less severe inflammation and has prognostic value in septic dogs.[11,12] Inflammatory cytokines decrease PON-1 and increase SAA transcription in cultured murine hepatocytes, offering a mechanism contributing to altered composition and function of HDL particles in the context of inflammation.[13] Concentrations of HDL are decreased in dogs with parvoviral enteritis and Leishmaniasis, and total cholesterol concentrations are prognostic in dogs with sepsis.[14-16] Thus, the decrease in PON-1 could alternatively reflect a loss of HDL.

Simple quantification of total plasma triglycerides (TG), cholesterol, and even total HDL and LDL provide an incomplete picture of lipid metabolism. As many as 10 subfractions of HDL and 7 subfractions of LDL exist in humans and subfractionation of lipoproteins is diagnostically and prognostically useful in humans with cardiovascular disease and insulin resistance.[17,18] Biochemical assays for human HDL and LDL fractions transfer poorly to dogs.[19] Some studies have used novel methods to evaluate inflammation-associated changes in lipid metabolism in dogs. For instance, a much larger HDL particle in dogs with babesiosis, compared to healthy controls, was demonstrated using a polyacrylamide gradient format and polystyrene beads.[20] Separately, the use of a bismuth sodium EDTA (NaBiEDTA) density gradient allowed for identification of a divergent lipoprotein in Miniature Schnauzers with normal total cholesterol and triglyceride concentrations and identified specific fractions were associated with primary hypertriglyceridemia.[21]

Investigating changes in lipoproteins concurrently with major APP, particularly those that are subject to transportation on lipoproteins, might aid to the understanding of the progression of inflammatory disease. A better understanding of lipid profiles in dogs with sepsis might aid illness severity assessment, enhance disease monitoring, enable identification of relapse or recurrence, and improve antimicrobial drug de-escalation. The objectives of this study were to evaluate a new immunoturbidometric assay for SAA (VET-SAA, Eiken Chemical Co, Ltd. Japan), determine the lipoprotein particle-size distribution (PSD) in dogs with sepsis, nonseptic inflammation (NSI), and healthy controls, and correlate dyslipidemic changes with SAA and CRP. We hypothesized that the new SAA assay would be more diagnostically useful than CRP in separating type of inflammation and that lipoprotein PSD would demonstrate unique profiles in each of the 3 groups.

2 | MATERIALS AND METHODS

2.1 | Animals

Unrelated ongoing studies were leveraged for collection of residual samples, with consent, from hospitalized client-owned animals that met inclusion criteria (Table 1). Blood samples were collected on presentation to the emergency department or at the time of transfer to the intensive care unit. Repeat laboratory submissions during hospitalization were performed at the discretion of the managing clinician. If serial sampling was ordered on the candidate dogs, the residual samples were identified and banked. Relevant data for the acute dog physiologic and laboratory evaluation illness severity scores ($APPLE_{full}$ and $APPLE_{fast}$) were collected on admission.[22] When initially recorded

TABLE 1 Enrollment criteria

1. Admission	Admission to ICU
2. Body weight	>3.0 kg
3. Age	1-9 years of age (inclusive)
4. SIRS criteria	2/4 met on admission or in first 24 hours
a. Heart rate	>140 beats/min
b. Respiratory rate	>20 breaths/min
c. Temperature	<100.2 or >103.1°F (<37.9 or >39.5°C)
d. WBC	<6.0 or >16.0 thousand/μL OR > 3% band neutrophils

Abbreviations: ICU, intensive care unit; min, minute; SIRS, systemic inflammatory response syndrome; WBC, white blood cell count.

TABLE 2 Comparison of demographics of enrolled dogs classified in the healthy (n = 22), nonseptic inflammation (n = 16), and septic inflammation (n = 25) groups

	Group			
Variable	**Healthy**	**Nonseptic**	**Septic**	***P* value**
Age in years	4.21 ± 1.71	5.83 ± 4.26	5.91 ± 3.87	.25[a]
Weight in kg	26.75 (14.2)	17.5 (26)	28.2 (14.05)	.18[b]
BCS out of 9	5 (0.5)	5 (2.5)	5 (0)	.94[b]
$APPLE_{(full)}$ score	n/a	22.3 ± 8.1	25.92 ± 10.1	.25[c]
Sex (%)				.01[d]
Intact female	0 (0.0)	5 (31.3)	0 (0.0)	
Neutered female	13 (59.1)	6 (37.5)	12 (48.0)	
Intact male	1 (4.6)	0 (0.0)	4 (16.0)	
Neutered male	8 (36.4)	5 (31.23)	9 (36.0)	

Note: Values are reported as mean ± SD, as median (interquartile range).
Abbreviation: BCS, body condition score.
[a]Welch's ANOVA.
[b]Kruskal-Wallis.
[c]Independent *t* test.
[d]Fisher's exact.

TABLE 3 Location of inflammation and specific disease etiology with relevant method of confirmation in the dogs with septic and nonseptic inflammation

Site	Abdominal/GI	Urogenital	Respiratory	Other
Septic	N = 9	N = 6	N = 8	N = 2
	Perforation: foreign body surgery (1) Abscess at gastropexy site *Staphylococcus pseudointermedius* (1) Peritonitis *Staphylococcus warneri* (1) *Escherichia coli* (1) *Streptococcus canis* (1) *Enterococcus faecium* (1) *Streptococcus bovis/equinus* (1) *Peptostreptococcus* (1) Liver abscess *Clostridium haemolyticum* (1)	Scrotal abscess (1) *Staphylococcus pseudointermedius* Pyometra *Staphylococcus pseudointermedius* (1) Surgery (2) Prostatic abscess *Streptococcus canis* (1) *Beta-hemolytic Escherichia coli* (1)	Aspiration pneumonia (5) Radiographs Infectious pneumonia PCR mycoplasma (2) Pyothorax *Streptococcus canis* (1)	Retrobulbar abscess imaging, cytology (1) Perirenal abcess *Streptococcus canis* (1)
Nonseptic	N = 11	N = 3	N = 1	N = 1
	Vomiting/diarrhea (4) Resolution to treatment Enteropathy Histopath (1) Ultrasound (1) Foreign body (1) Surgery Splenic infarction (1) Histopathology Intussusception (1) Surgery, negative cultures Gastric mast cell tumor (1) Histopath Abdominal mass (1) Ultrasound	Prolapsed vulva (1) Vestibulovaginal stenosis (1) Acute kidney injury (1) Negative blood cultures	Rhinitis (1) Cultures negative	Osteomyelitis (1) Blood cultures Negative

vital signs precluded APPLE scoring (eg, panting), the next available value from the medical record was used, typically within 4 hours and always within 24 hours per APPLE guidelines.[22] Dogs were diagnosed with septic or nonseptic systemic inflammation based on cytological identification of an infectious organism, bacterial or fungal culture results, PCR confirmation of an infectious etiology, imaging results, or

TABLE 4 Serum amyloid A assay method validation, precision studies

				Controls[a]	
Sick dog samples	LOW	Medium	High	Low	High
Intra-assay	24.06 (0.52, 0.02)	156.4 (2.55, 0.02)	318.02 (4.83, 0.02)	9.33 (0.36, 0.04)	49.07 (1.14, 0.02)
Inter-assay	0.64 (0.4, 0.53)	161.1 (3.33, 0.02)	334.06 (10.7, 0.03)	9.73 (0.52, 0.05)	50.35 (1.08, 0.02)

Note: Values represent the average (SD, coefficient of variation) mg/L of 10 replicates assayed with the same run (intra-assay) or in replicates runs on sequential days (inter-assay).

[a]Controls are the manufacturer provided reagents and the low, medium, and high patient samples are pooled to achieve the concentrations spanning the assay working range.

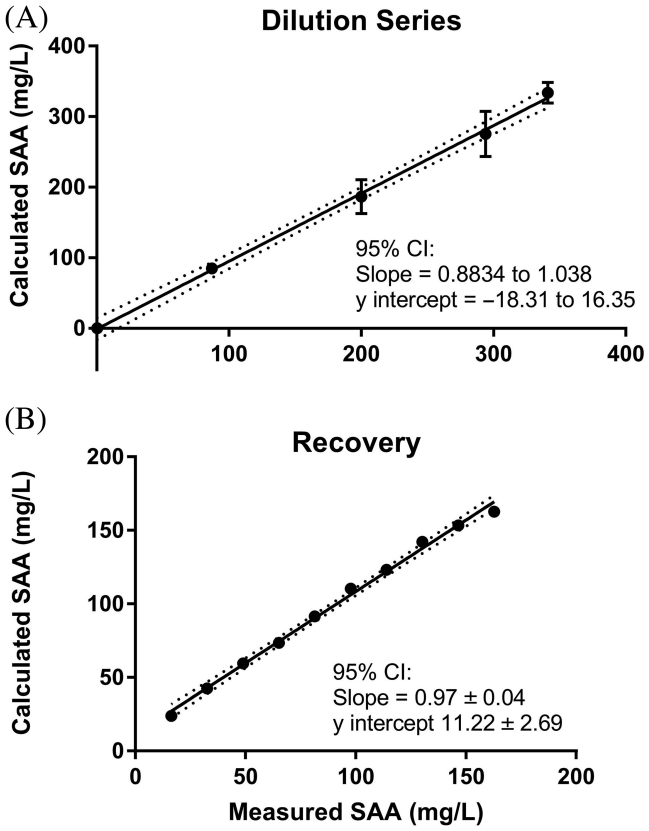

FIGURE 1 Linearity under dilution and recovery experiments. A high concentration sample (318 mg/L) was run undiluted and mixed with a low concentration sample (0.32 mg/L) at the following ratios: 1 : 3, 1 : 1, 3 : 1, and the low concentration sample run neat. Samples were run in triplicate. Data are expressed as the mean, SD, and 95% CI (A). A pooled sample (163 mg/L) was added in 10% increments to sample diluent. Samples were run in triplicate. Data are expressed as the mean, SD, and 95% confidence interval (CI; B)

surgical findings. Respective primary clinicians determined all aspects of case management. Cholesterol results were available to clinicians as part of standard serum chemistry profiles. Serum amyloid A results were available when specifically ordered by clinicians. Clinical pathology data collected from each dog included: CBC; serum biochemistry panel with electrolytes; measurements of CRP, SAA, triglyceride and cholesterol concentrations, HDL, LDL; and fractionation of HDL and LDL via nondenaturing linear polyacrylamide gel electrophoresis (Lipoprint, Quantimetrix, Redondo Beach, California). Healthy dogs were recruited from staff-owned pets and animals presented to the hospital for wellness examinations. Healthy dogs were eligible for enrollment if they weighed >3 kg, had no chronic or recent illness, and had received no medications other than preventative in the preceding 3 months. To avoid age-associated increases in serum lipids and potential juvenile parasitism, age was restricted to 1 to 9 years.[23] Dogs were determined to be healthy based on history, physical examination, CBC, and serum biochemistry profile results. Samples were collected under Institutional Animal Care and Use Protocols #: 2007-0146 and 2014-0052 (healthy dogs) and 2014-0053 (ill dogs).

2.2 | Laboratory methods

All clinical pathology testing was performed by an American Association of Veterinary Diagnostic Laboratories accredited laboratory. Hematology and chemistry analyzers in the laboratory were evaluated daily using commercial quality control materials and calibrated as needed. The laboratory participates in 2 external quality assurance programs. Automated CBC and hematologic analysis was performed using an ADVIA 2120 analyzer (Siemens; Munich, Germany). Medical technologists performed blood smear evaluation on all dogs to confirm automated results, evaluate cell morphology, and confirm platelet estimates. Blood smears were reviewed by boarded clinical pathologists when indicated based on internal quality assurance protocols. Plasma appearance was evaluated and total protein was provided by refractometer. Serum biochemistry profiles were performed using an automated wet chemistry analyzer (Roche Cobas 6000 series 501c3; Basel, Switzerland). Analytes included: sodium, potassium, chloride, bicarbonate, anion gap, urea, creatinine, calcium, phosphorus, magnesium, total protein, albumin, globulins, glucose, aspartate aminotransferase, alkaline phosphatase, γ-glutamyl transferase, total bilirubin, cholesterol, TG, creatinine kinase, amylase, iron, total iron binding capacity, and % iron saturation. Immunoturbidometric assays were used to quantitate CRP and SAA concentrations. Analytical validation of the CRP assay (Gentian Diagnostics AB; Sweden) has been previously reported,[24] and internal method performance was verified when the assay was introduced in the laboratory. Validation of the SAA assay that employs monoclonal antibodies was performed as part of the current study and included determination of intra- and inter-assay

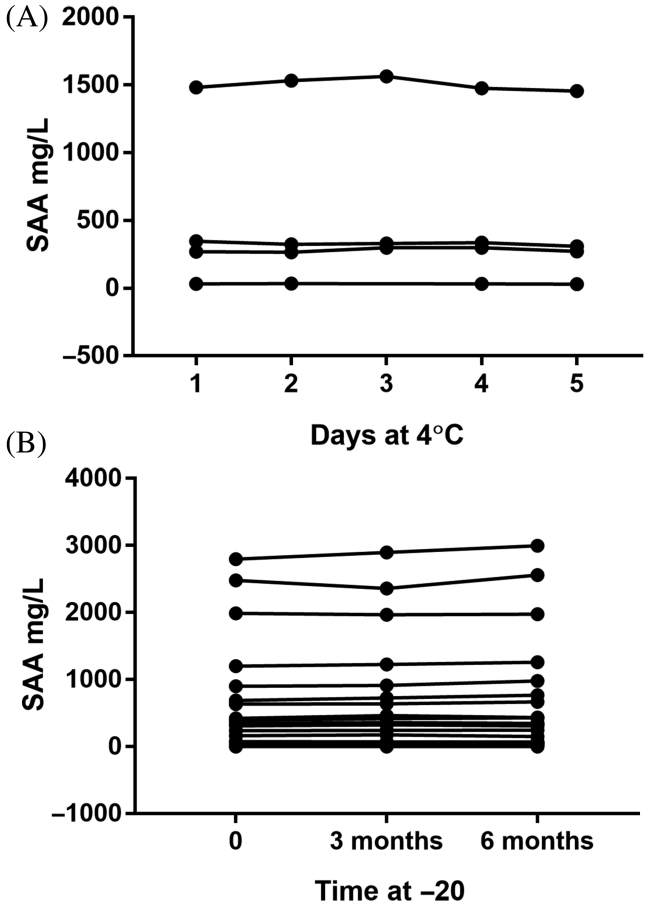

FIGURE 2 Sample stability. Serum from 4 dogs was stored at 4°C and analyzed on 5 consecutive days. Individual data points are shown. One replicate is missing (3 days of storage for lowest samples, fell on a day the laboratory was closed). Coefficient of variations for each data set over time were less than 5%, with a single exception (sample 2, values equal to 2.0, 0.5, and 0.6) (A). Twenty separate dog samples were stored at −20°C and re-analyzed after 3 and 6 months. Individual data points are shown. Coefficient of variations for each data set over time were less than 5% (B). No differences over time were found (repeated measures ANOVA). SAA, serum amyloid A

precision, linearity on dilution, spiking and recovery, limit of detection (LOD) and limit of quantification (LOQ), and an evaluation of sample storage at 4°C and −20°C.

2.3 | LDL and HDL Subfractionation

Plasma samples were analyzed using commercial kits (cat 48-7002 and 48-9002, Quantimetrix), according to the manufacturer's instructions. The samples were briefly thawed at room temperature and after removal of storage buffer from the top of the gel tube, 25 μL of plasma was loaded into the precast linear polyacrylamide gels. Loading gel (200 μL for the LDL gel, 300 μL for the HDL gel) was added, tubes covered, and inverted gently to mix samples with loading gel solutions. The gels were photopolymerized for 30 minutes and electrophoresed for 1 hour at 3 mA per tube in the provided buffers (tris[hydroxymethyl]aminomethane and boric acid). Dog samples were run alongside a control human plasma sample provided by the manufacturers (LipoSep) for quality assurance purposes.

2.4 | Densitometry

After electrophoresis, tubes were scanned in a flatbed scanner equipped with a cold-cathode fluorescent lamp (ArtixScan M1, Microtek International Inc, Hsinchu City, Taiwan), and analyzed using the Image SXM program (Dr Steve Barrett, University of Liverpool) (Lipoware, Quantimetrix). The software converts measured band intensity to an area under the curve (AUC) and then a relative percentage value, using known concentrations of human LDL and HDL enabling reporting concentrations of each subfraction in mg/dL. Routine biochemical tests to quantify human HDL and LDL do not transfer to canine species; therefore, we used a default value of 100 mg/dL for LDL and HDL and generated a percentage for each subfraction.

TABLE 5 Comparison of acute phase protein (APP) concentrations and major serum lipid classes (total cholesterol and triglyceride concentrations, LDL, and HDL) across study groups

Variable	Healthy	Nonseptic	Septic	*P* value	Significant post hoc
CRP	4.9 (0)[a]	91.1 (88.6)	133.2 (91.6)	<.0001	H-NS, H-S
SAA	0 (0)[b]	201 (436)	460 (886)	<.0001	H-NS, H-S
Triglycerides	52.5 (28)	53 (14)	84 (87)	.03	None
Cholesterol	230.5 (54)	189 (49)	218 (91)	.08	n/a
HDL	71.7 (8.4)	62.25 (13.3)	55.4 (28.5)	.0003	H-NS, H-S
LDL	14 (8)	22.5 (14)	27.5 (18)	.0005	H-S

Notes: Values reported as median (interquartile range). Serum amyloid A (SAA) and C-reactive protein (CRP) (mg/L), cholesterol and triglycerides (TG) (mg/mL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) as % of total. *P*-values from Kruskal-Wallis test with 2 degrees of freedom. Post hoc testing via Dunn's test.

Abbreviations: H, healthy, NS, nonseptic; S, septic.

[a]All except 1 value fell below the detection threshold (<5.0) and were set to 4.9.

[b]Nineteen of 24 had values of 0.

2.5 | Statistical methods

Sample size calculations were performed using data from previously published studies evaluating lipoproteins in humans with sepsis. A 1-way ANOVA with 3 equal group weights was performed with α of .05 and $(1 - \beta)$ of .8 (SAS 9.4, SAS, Cary, North Carolina) using the following mean values for total cholesterol 191 mg/dL (healthy controls), 170 mg/dL (hospitalized, non-septic dogs), and 132 mg/dL (hospitalized, septic dogs),[25] using the largest SD reported to provide the most conservative sample size estimate. Calculations suggested a total of 39 dogs (13 per group) were required with an actual power of 0.82. Data were evaluated for normality by visual inspection of Q-Q plots and the Shapiro-Wilk test. Homogeneity of variances was determined by Levene's test. Parametric data with homogeneous variances were analyzed with 1-way ANOVA and Tukey's post hoc tests, while parametric data with inhomogeneous variances were analyzed with Welch's ANOVA and Games-Howell post hoc tests. Non parametric data were analyzed using the Kruskal-Wallis test with Dunn's post hoc tests. $APPLE_{full}$ scores were compared between the SI and NSI groups using an independent *t* test after confirming equal variances using a Satterthwaite test. Bivariate associations between $APPLE_{full}$ scores and both total cholesterol concentration and HDL were evaluated via linear regression, with normality assessed via inspection of studentized residual Q-Q plots and homoscedasticity assessed by inspection of residuals plotted against fitted values. For models in which 1 or both assumptions were violated, transformation of the response variable was attempted with refitting of the model. If standard transformations also violated the assumptions of linear regression, Spearman's correlation coefficient was calculated. Receiver-operating characteristic curves were generated using the PRISM software version 9.

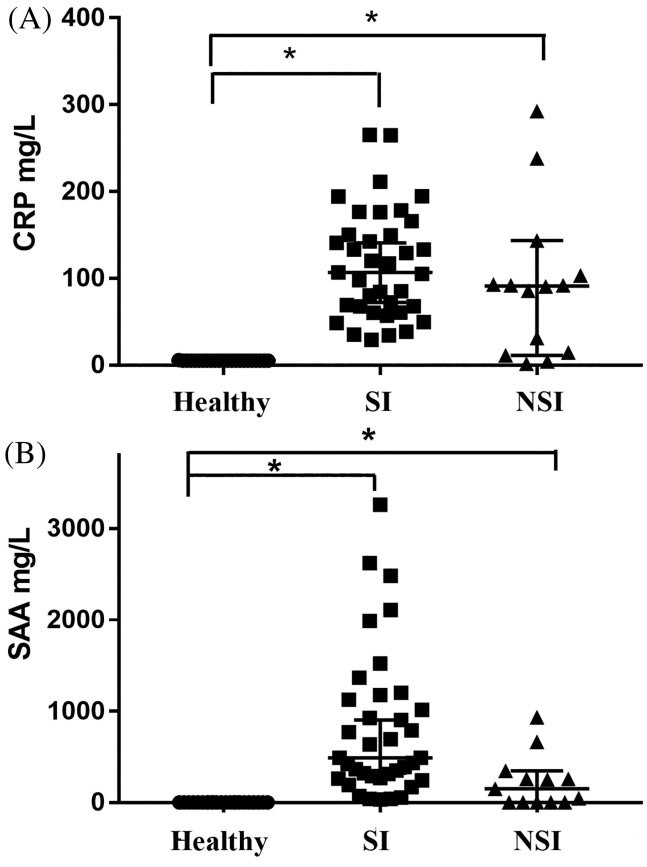

FIGURE 3 Serum amyloid A (SAA) and C-reactive protein (CRP) are both increased in all dogs with inflammation tested. Healthy n = 22, 25 septic inflammation (SI) n = 25, nonseptic inflammation (NSI) n = 16. *Different from each other, $P < .05$

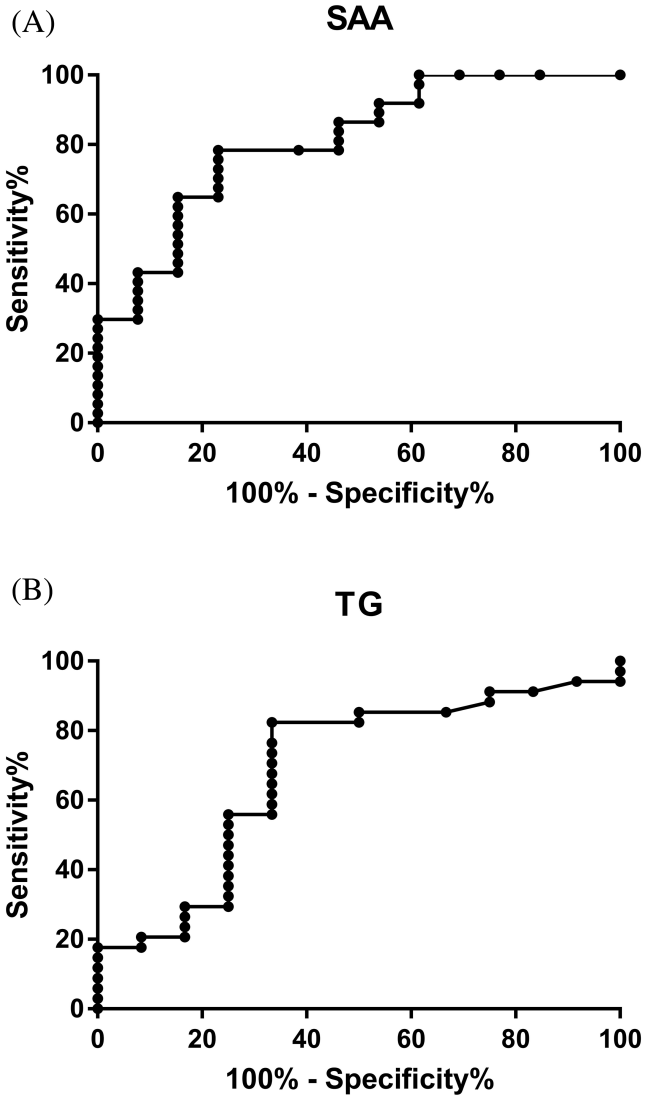

FIGURE 4 Serum amyloid A (SAA) but not triglycerides (TG) are diagnostically useful in predicting septic inflammation (SI). Receiver-operating characteristic curves for SAA (mg/L) and triglyceride concentrations (mg/dL) in dogs with inflammation. Sensitivity and specificity determined relative to separating SI from non-septic inflammation dogs

TABLE 6 Comparison of serum amyloid A (SAA), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) concentrations across study groups, outlier excluded

	Healthy	Nonseptic	Septic	*P* value	Significant post hoc
SAA	0 (0)	97.0 (302.1)	770.0 (836.0)	<.0001[a]	H-NS, H-S, NS-S
HDL	72.4 ± 7.2	62.6 ± 8.3	52.7 ± 16.9	<.0001[b]	H-NS, H-S, NS-S
LDL	14.4 ± 5.6	21.0 ± 7.8	29.6 ± 14.6	.001[b]	H-S, NS-S

Note: Values reported as mean ± SD or as median (interquartile range).
Abbreviations: H, healthy, NS, nonseptic; S, septic.
[a]Kruskal-Wallis test with Dunn's post hoc analyses.
[b]Welch's ANOVA with 2 degrees of freedom and post hoc testing via Games-Howell test.

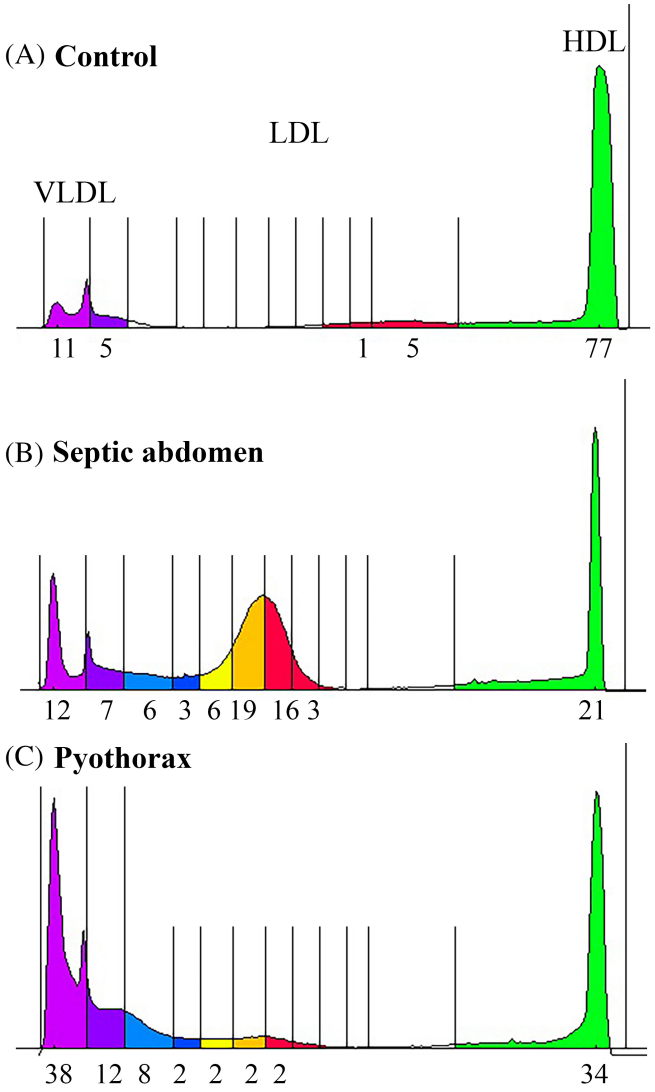

FIGURE 5 Representative lipoprotein densitometry plots from a healthy control dog (A), a dog with septic inflammation (SI) because of a foreign body-induced intestinal perforation (B), and a dog with a septic pyothorax (C). The numbers along the X-axis represent the percent of the total area under the curve. HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein

3 | RESULTS

3.1 | Animals

Samples from 63 dogs were analyzed, from 25 dogs with sepsis, 16 dogs with nonseptic systemic inflammation, and 22 healthy control dogs. The most commonly represented breeds were mixed breed (25), Labrador Retrievers (6), Great Dane (6), German Shepherd (4), and 2 each of Boston Terrier, Golden Retriever, and American Pit Bull Terrier. Descriptive statistics and additional clinical details are summarized in Tables 2 and 3, respectively. No statistically significant differences were found between groups with respect to age, weight, or body condition score. $APPLE_{full}$ scores were not different between SI and NSI groups ($P = .19$). The sex distribution between groups differed ($P = .01$), with only the NSI and SI groups differing after Bonferroni correction ($P = .02$). No significant difference was found in the anatomical origin (eg, GI vs respiratory system) of the disease represented in the NSI vs SI group.

3.2 | SAA assay performance characteristics

Intra-assay precision was determined by running low and high control materials and 3 pooled dog samples targeting low, medium, and high concentrations 10 times in a single day. Inter-assay precision was determined by running controls and the 3 pooled dog samples on 10 consecutive days (Table 4). Linearity under dilution was determined by measure of a high and low concentration sample mixed over a range of dilutions (high undiluted, 1 : 3, 1 : 1, 3 : 1, and low) maintaining serum as the matrix. Samples were run in triplicate. Spiking recovery experiments were performed by addition of a pooled high concentration sample added to dilution buffer. All performance studies yielded acceptable results (Figure 1). The LOD and LOQ were determined using the following calculations:

LOD: Method 1: SD blank + 3 (SD blank) = 0.947 mg/L

Method 2: Mean of blank + 1.65 × SD blank + 1.65 × SD low sample = 0.979 mg/L

LOQ: Mean (low sample) + bias + 2 × SD (low sample) = 9.674 mg/L

Samples from 4 dogs distributed across the range of the assay were stored at 4°C and analyzed on 5 consecutive days. Samples from 20 dogs, again spanning the full range of results, were stored in single-thaw aliquots for 3 and 6 months at −20°C. There was no change over the time periods evaluated (Figure 2).

3.3 | APP and serum biochemistry results

Creactive protein and SAA concentrations were below detection limits of the assays in all healthy dogs. In bivariate analyses, median CRP,

TABLE 7 Comparison of lipoprotein subfractions measured by high-resolution polyacrylamide electrophoresis across study groups

Variable	Group			*P* value	Significant post hoc
	Healthy	Nonseptic	Septic		
Mid fractions	1.8 (0.54)	2.13 (0.82)	2.45 (0.59)	<.007[a]	H-S
VLDL	12.2 /−5.3[a]	14.5 /−7.0	15.4 /− 9.3	.35[b,c]	n/a
LDL 1-2	0.0 (0.0)	1.3 (4.1)	3.6 (6.4)	<.0001[a]	H-NS, H-S
LDL 3-7	7.8 (5.8)	12.2 (9.4)	10.9 (8.3)	.23[b]	n/a
HDL 7	8.1 (2.5)	7.8 (3.4)	7.6 (4.1)	.87	n/a
HDL 8	10.5 (3.2)	9.1 (5.7)	7.8 (5.5)	.14	n/a
HDL 9	9.5 (2.7)	7.7 (5.7)	5.3 (5.8)	.01[a]	H-S
HDL 10	4.6 (3.9)	4.0 (5.1)	2.1 (3.7)	.06	n/a

Abbreviations: H, healthy; NS, nonseptic; S, septic.
Values reported as mean % of total lipoprotein ± SD or as median (interquartile range).
[a]Tukey's post hoc.
[b]Kruskal-Wallis test with Dunn's post hoc analyses.
[c]Welch's ANOVA with 2 degrees of freedom and post hoc testing via Games-Howell test.

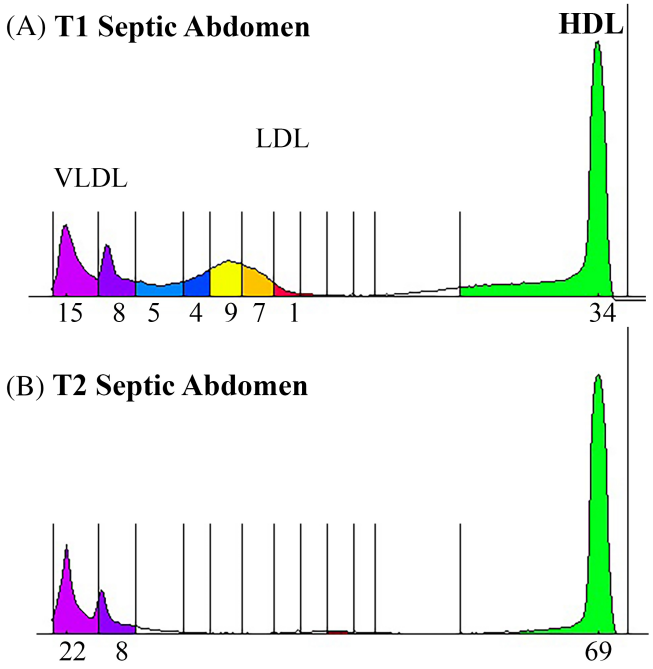

FIGURE 6 Example densitometry plots showing resolution of inflammation corresponding to the disappearance of large LDL species (peaks in orange and red). Lipoprotein electrophoretogram at the time of admission (T1; A) and 3 days after surgery was performed to remove devitalized intestinal tissue resulting from ingestion of a foreign body (T2; B). HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein

SAA, TG, cholesterol, HDL, and LDL all differed between groups (all $P < .05$) (Table 5). Post hoc analyses indicated that both CRP and SAA were significantly lower in healthy dogs than in NSI and SI dogs. The range of SAA concentrations extended to 3620 mg/L, whereas the highest measured CRP concentration was 264.4 mg/dL (Figure 3). Triglycerides were significantly higher in septic dogs than both healthy and nonseptic dogs (Table 5).

Receiver-operating characteristic curve analysis suggested that SAA was discriminating for SI compared to NSI (AUC 0.81 [95% confidence interval [CI] 0.67-0.95], $P = .001$). Using a cutoff of >677.5 mg/L, SAA was 43.2% sensitive and 92.3% specific for SI and correctly identified 17/18 dogs (94%) as having SI. Triglycerides were not useful for distinguishing dogs with NSI from dogs with SI (AUC 0.69 [95% CI 0.49-0.89], $P = .06$; Figure 4).

Multivariable logistic regression with stepwise selection and entry threshold of $P < .2$ was performed to assess whether HDL, LDL, TG, or total cholesterol was predictive of SI (vs NSI) with covariate adjustment (age, weight, and body condition score). A separate covariate-adjusted model was fit for each lipid component; each model incorporated all 2-way interaction terms. Sex could not be assessed as a covariate because of complete or quasi-complete separation. Triglycerides, HDL, and cholesterol all failed model entry at the specified threshold; the model incorporating LDL found a covariate-adjusted odds ratio (OR) estimate for HDL of 1.048 (95% CI 0.98-1.12, $P = .16$), with weight as the sole covariate (OR 1.048 [95% CI 0.98-1.12], $P = .07$).

One subject in the NSI group was an extreme outlier (determined by a *Z*-score with a threshold of 2.5) and an influence point with respect to SAA (>3000); so, subanalysis was performed omitting this observation. Dropping the outlier primarily affected HDL and LDL, resulting in normal distributions within each disease group; in the re-analysis, median HDL and LDL were again found to differ between groups (both $P \leq .001$; Table 6). Median HDL was significantly greater in the healthy group compared to that in both the NSI and SI groups, and significantly greater in the NSI group compared to that in the SI group. Median LDL was lower in both the healthy and NSI groups compared to that in the SI group.

A simple (bivariate) linear regression found no significant association between $APPLE_{full}$ score and total cholesterol ($P = .49$), HDL ($P = .54$), or CRP ($P = .58$). After square root transformation of SAA, which results in normal residuals (Satterthwaite test $P = .18$),

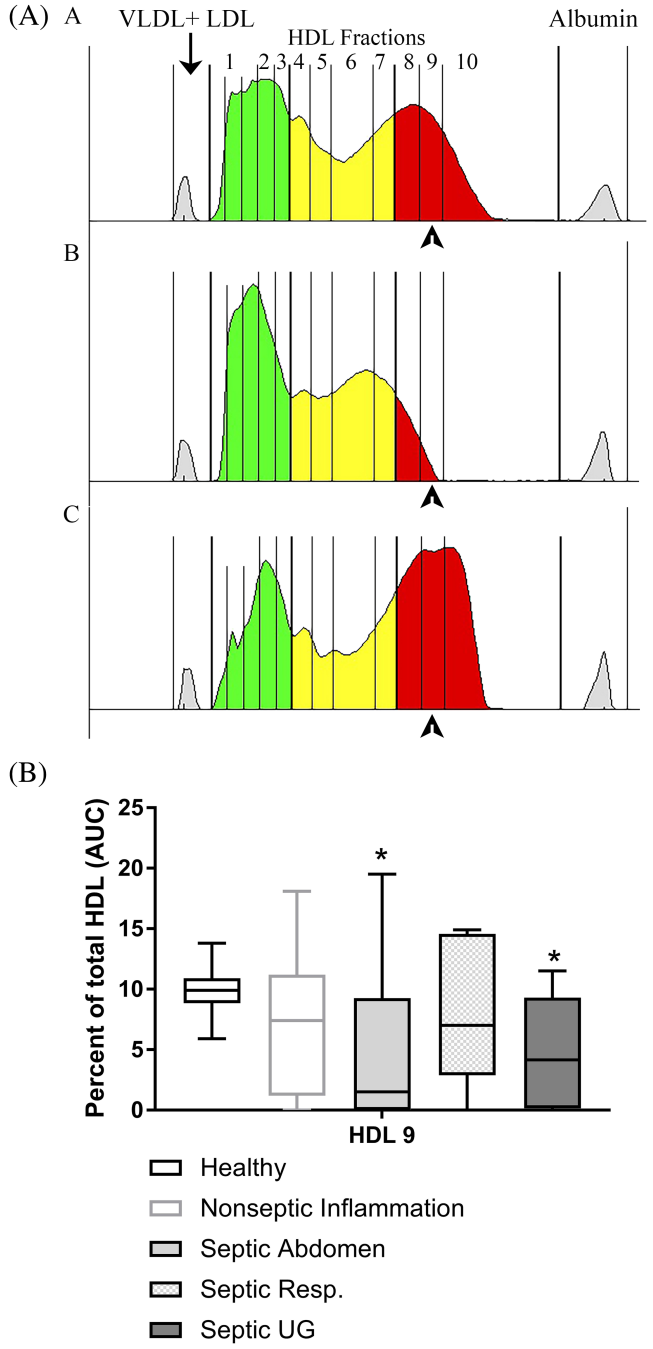

FIGURE 7 Representative HDL fractionation from the 3 groups of dogs (A). A plot from a healthy dog (letter A), and 2 dogs in the septic inflammation (SI) group; 1 with a septic abdomen (letter B), and the second a septic respiratory infection (letter C) are shown. The arrows indicate HDL fraction 9. Summative analysis of HDL subspecies 9 in all dogs (B). Dogs with SI were further subdivided by the location of the septic process. Lines indicate the median values, whiskers represent the Tukey post hoc test, and * indicates different from each other $P < .05$. HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein

$APPLE_{full}$ score and the square root of SAA were weakly linearly correlated ($r^2 = 0.12$, parameter estimate [beta] = 0.57, SE = 0.25, $t = 2.29$, $P = .03$).

3.4 | Analysis of lipoprotein subfractions

High-density lipoprotein was significantly greater in healthy dogs compared to that in both NSI and SI dogs, while LDL was lower in healthy dogs vs SI dogs (Figure 5; Table 6). Healthy dogs had lower amounts of LDL fractions 1 and 2 compared to NSI and SI dogs (Table 7). Four dogs with SI because of septic peritonitis displayed marked increases in the larger LDL subfractions (Figure 5A). Although not a primary objective of the study, these dogs were followed over time and repeat analysis showed a disappearance of these fractions upon disease resolution in all 4 cases (Figure 5B). Dogs with SI had smaller amounts of HDL subfraction 9 compared to healthy dogs (Table 7; Figure 6). The average proportions of other lipoprotein subfractions were not significantly different across groups (Figure 7).

4 | DISCUSSION

Measurement of major acute phase protein concentrations is a crucial means to identify inflammation and to monitor progression or resolution. The pathophysiological interplay between inflammation and lipid metabolism suggests that lipid fractions might serve as biomarkers of inflammation and potential therapeutic targets. In this study, a new immunoturbidometric reagent for SAA measurement was validated, and had diagnostic useful properties not demonstrated for CRP. Evaluation of lipoprotein PSD identified electrophoretic shifts in lipoproteins that warrant further investigation. Study results also underpin the minimal utility of surrogate markers, including total cholesterol and triglyceride concentrations, in separating septic from NSI.

The SAA assay met desired performance characteristics and was deemed reliable for clinical use in dogs. All healthy dogs had CRP concentrations below the assay LOQ, consistent with previous reports.[24] The same was also true for SAA concentrations. In dogs with bacterial pneumonia, CRP and SAA are increased but the magnitude of increase does not correlate with disease severity.[26] In our study, SAA was different between the SI and NSI groups after exclusion of a single outlier, whereas CRP was not. Serum amyloid A was weakly correlated with $APPLE_{full}$ score. The range of SAA values extended approximately 3-fold higher than the range of CRP values, although this range is not as broad as that reported in horses.[27] Regardless, this feature of the assay might facilitate improved dog monitoring and the development of numerical cutoffs to guide therapeutic decision-making including antimicrobial drug discontinuation as has been described for CRP.[26] In addition to its role as a biomarker of inflammation, SAA binds a number of proinflammatory receptors and activates proinflammatory pathways, and its decline is proposed to be part of a feedback loop using pro-resolving (M2 type) macrophages.[28] Serum concentrations of inflammatory cytokines known to induce SAA synthesis are higher in neonates with culture-proven sepsis compared to those with culture-negative sepsis.[29,30] Microbial products could be contributing to this association. These functional roles of SAA in modulating inflammation underpin the potential use of SAA in

prognosticating severity and resolution disease. Clinical decision limits for both CRP and SAA to identify postoperative infections in dogs after tibial plateau leveling osteotomy have also been published.[31] However, the dogs in that study all had clinical signs indicative of infections so the true sensitivity and specificity for identifying occult infections is not yet established. The potential use of SAA in monitoring disease resolution requires further evaluation as temporal trends were not investigated in the current study.

Most of the changes in lipid profiles in dogs with inflammatory diseases were consistent with the existing literature, whereas the identification of novel subfractions warrants further investigation. Proinflammatory cytokines, such as interleukins 1 and 6, tumor necrosis factor, platelet-derived growth factor, and transforming growth factor beta, alter lipid metabolism and decrease total cholesterol concentrations.[32,33] Thus, the changes in total cholesterol in the current study was as expected. Decreased cholesterol is not a feature of all types of inflammation in the dog, however, since most dogs with pancreatitis have normal serum cholesterol and triglyceride concentrations, but higher LDL and lower very low-density lipoprotein (VLDL) and HDL fractions compared to controls.[34]

The negative correlation between HDL and SAA in dogs is also consistent with reports in humans and mice. In these species, increases in inflammatory cytokines promote synthesis of SAA and decrease synthesis of negative APP including the apolipoproteins (Apo) and enzymes necessary for formation of HDL. Serum amyloid A further complicates dyslipidemia by displacing ApoA-I from HDL molecules, resulting in the formation of acute phase HDL which has inferior antioxidant properties.[35,36] The impact of SAA on the function of canine HDL is unknown at this time. The higher concentration of TG in dogs with SI paralleled an increased in LDL and aligns with the reported composition of LDL in dogs.[37] Previous studies have largely relied on agarose-based methodology which semiquantifies only the 4 major lipoprotein classes; chylomicrons, VLDL, LDL, and HDL.[38,39] Methods using gel- or liquid-based chromatography identify additional fractions but require specialized equipment that are not broadly available.[21] The commercial assay used in the present study allowed for the identification of specific lipoprotein changes that were different across groups.

Lipoproteins and phospholipids regulate oxidative pathways and can be intermediates in the production of inflammatory mediators and resolvins that suppress inflammation.[40,41] Additionally, studies have shown that HDL composition changes in human sepsis.[42,43] The links between lipoprotein composition and immunomodulatory function support further investigation into these particular subfractions in dogs.

Our study has limitations. Factors that can impact lipid metabolism including diet, age, and body condition were not controlled for.[44,45] Additionally, underlying endocrinopathies also known to impact lipid metabolism including hypothyroidism, diabetes mellitus, and hyperadrenocorticism were not evaluated in the dog population.[46] As with many veterinary studies, sample size was small and the selection criteria for dogs in the study were skewed toward clinically recognizable inflammation. Additional studies evaluating the diagnostic and prognostic utility of the SAA assay in diseases with mild to minimal inflammation are warranted. To facilitate consistency across future studies and the ability of other groups to replicate this work, the algorithm for determining fraction cutoffs was not modified from the manufacturer's recommendations, but was therefore not optimized for the dogs in the present study.

The wider range of the new SAA assay as compared to its predecessors, higher concentrations of SAA found in dogs with sepsis, and potential role of SAA as modulator of macrophage function in the resolution of inflammation, all support the need for longitudinal studies in dogs with SIRS using the assay. Routinely measured markers of lipid metabolism, including total TG and cholesterol concentrations, have limited diagnostic utility. The clinical utility of the alterations in LDL and HDL PSD is not clear at this time. The changes in size of LDL and HDL particles likely reflect changes in composition, which subsequently might alter their function. An improved understanding of lipid metabolism and PSD in dogs with SIRS and perhaps more specifically SI could aid in evaluating illness severity, after the course of disease, identifying emergence of secondary disease processes, and determining the appropriate timing of antibiotic de-escalation. Finally, as dietary and pharmacological approaches can both modify lipid metabolism, this might offer novel avenues to explore therapeutically.

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CONFLICT OF INTEREST DECLARATION

Eiken Chemical Company supplied their SAA reagent to us at no cost. We have shared our validation data with Eiken Chemical Company. The agreement we put in place gave Cornell University full ownership of the data and the right to publish the results. The article is fully independent of Eiken's influence.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Approval was not required directly for this study as no samples were collected for the purpose of this study. Dr Goggs had multiple ongoing studies (all with IACUC approvals) and we used residual samples from these studies. Cornell University has a method validation IACUC in place.

HUMAN ETHICS APPROVAL DECLARATION

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

ORCID

Erica Behling-Kelly https://orcid.org/0000-0001-9189-4238
Robert Goggs https://orcid.org/0000-0001-7446-6987

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