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Association of acute *Babesia canis* infection and serum lipid, lipoprotein, and apoprotein concentrations in dogs

Zorana Milanović[1] | Jelena Vekić[2] | Vladimir Radonjić[3] | Anja Ilić Božović[1] | Aleksandra Zeljković[2] | Jelena Janac[2] | Vesna Spasojević-Kalimanovska[2] | Jesse Buch[4] | Ramaswamy Chandrashekar[4] | Žanka Bojić-Trbojević[5] | Ljiljana Hajduković[5] | Mary M. Christopher[6] | Milica Kovačević Filipović[1]

[1]Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Serbia

[2]Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia

[3]Small animal practice "Petrovac", Beograd, Serbia

[4]IDEXX Laboratories, Westbrook, Maine

[5]INEP - Institute for the Application of Nuclear Energy, University of Belgrade, Zemun, Serbia

[6]Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California, Davis, California

Correspondence
Zorana Milanović, Department of Pathophysiology, Faculty of Veterinary Medicine, University of Belgrade, Bulevar oslobođenja 18, 11000 Belgrade, Serbia.
Email: zoranamilanovic89@gmail.com

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Abstract

Background: *Babesia canis* infection induces a marked acute phase response (APR) that might be associated with alteration in lipid and lipoprotein metabolism and disease prognosis.

Hypothesis: Dogs with *B. canis*-induced APR develop dyslipidemia with altered lipoprotein concentration and morphology.

Animals: Twenty-nine client-owned dogs with acute *B. canis* infection and 10 clinically healthy control dogs.

Methods: Observational cross-sectional study. Serum amyloid A (SAA) was measured using ELISA. Cholesterol, phospholipids, and triglycerides were determined biochemically. Lipoproteins were separated using agarose gel electrophoresis. Lipoprotein diameter was assessed by polyacrylamide gradient gel electrophoresis; correlation with ApoA-1 (radioimmunoassay) and SAA was determined.

Results: Dogs with *B. canis* infection had a marked APR (median SAA, 168.3 μg/mL; range, 98.1-716.2 μg/mL) compared with controls (3.2 μg/mL, 2.0-4.2 μg/mL) ($P < .001$). Dogs with *B. canis* infection had significantly lower median cholesterol (4.79 mmol/L, 1.89-7.64 mmol/L versus 6.15 mmol/L, 4.2-7.4 mmol/L) ($P = .02$), phospholipid (4.64 mmol/L, 2.6-6.6 mmol/L versus 5.72 mmol/L, 4.68-7.0 mmol/L) ($P = .02$), and α-lipoproteins (77.5%, 27.7%-93.5% versus 89.2%, 75.1%-93.5%) ($P = .04$), and higher

Abbreviations: ApoA-1, apolipoprotein A-1; APR, acute phase response; CETP, cholesterol ester transfer protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LPS, lipopolysaccharide; PAGGE, polyacrylamide gradient gel electrophoresis; PCR, polymerase chain reaction; SAA, serum amyloid A; SR-B1, scavenger receptor, class B type 1; TRL, triglyceride-rich lipoproteins; VLDL, very low-density lipoprotein.

ApoA-1 (1.36 U, 0.8-2.56 U versus 0.95 U, 0.73-1.54 U) concentrations (P = .02). Serum amyloid A correlated with high-density lipoproteins (HDLs) diameter (rho = .43; P = .03) and ApoA-1 (rho = .63, P < .001).

Conclusions and Clinical Importance: Major changes associated with *B. canis*-induced APR in dogs are related to concentration, composition, and morphology of HDL particles pointing to an altered reverse cholesterol transport. Parallel ApoA-1 and SAA concentration increase is a unique still unexplained pathophysiological finding.

KEYWORDS

acute phase response, apolipoprotein A-1, high-density lipoprotein, lipoprotein diameter, serum amyloid A

1 | INTRODUCTION

The acute phase response (APR) is induced by tissue injury and governed by a network of neuro-immuno-humoral signals that enable rapid adaptation, elimination of harmful agents, and repair of damaged tissue. One of the best described markers of an APR is an increase in serum concentrations of acute phase proteins, such as serum amyloid A (SAA). Acute phase responses also induce alterations in lipid metabolism that can lead to important changes in plasma lipids (cholesterol, phospholipids, and triglycerides) and in the concentration, morphology (diameter), and composition of lipoproteins.[1] Some changes, such as a decrease in high-density lipoprotein (HDL)-cholesterol, are related to poor prognosis and death in septic humans and rodents.[2]

In humans and rodents, the APR is characterized not only by a decrease in HDL, but also by a decrease in serum apolipoprotein (Apo) A-1, the main apoprotein of HDL, suggesting altered reverse cholesterol transport.[3] Reverse cholesterol transport is a multistep process leading to cholesterol transfer from peripheral tissues to the liver, where uptake is mediated by the scavenger receptor, class B type 1 (SR-B1).[1] ApoA-1 is indispensable for cholesterol efflux and esterification, and for the remodeling of HDL particles.[4] Furthermore, in acute inflammation, HDL becomes enriched with SAA, resulting in further modification of its morphology and diameter.[5,6] The overall consequence of an APR is decreased cholesterol efflux, altered HDL morphology, and decreased cholesterol uptake through SR-BI in the liver.[1]

The influence of the APR on lipids and lipoproteins in dogs is poorly understood. In dogs, the majority of serum cholesterol is contained within HDL and involved in reverse cholesterol transport.[7] Canine HDL particles form distinct α1 and α2 bands in electrophoretic gels that correspond quantitatively to HDL-2 and HDL-1.[8,9] Dogs lack cholesterol ester transfer protein (CETP) activity, which in humans is involved in transferring cholesteryl esters from HDL to low-density lipoproteins (LDLs).[10] These species differences might affect changes in lipids and lipoproteins in dogs with inflammatory disease.

Babesia canis causes an important tick-borne disease that induces a typical APR in dogs, with a major increase in SAA.[11] Total cholesterol and HDL-cholesterol levels are lower at the time of presentation than after treatment with imidocarb-dipropionate,[12] and ApoA-1 concentration is higher in dogs with acute babesiosis.[13] However, in those studies, the method used for HDL cholesterol was not recommended for use with canine serum,[14] and the ApoA-1 results, obtained via mass spectrometry, were unusual and unexplained. Furthermore, changes in lipids and lipoproteins have not yet been investigated in conjunction with SAA levels in dogs with babesiosis, nor have changes in lipoprotein electrophoretic patterns or lipoprotein morphology been examined.

We hypothesized that dogs with *B. canis*-induced APR develop dyslipidemia with altered lipoprotein concentration and morphology, compared with healthy dogs. Our aims were to (1) measure serum lipids, SAA and ApoA-1 concentrations concurrently in dogs with acute *B. canis* infection and to compare the results with those in clinically healthy controls; (2) evaluate differences in lipoprotein types via electrophoresis; and (3) evaluate lipoprotein diameter and its correlation with SAA and ApoA-1 concentrations.

2 | MATERIALS AND METHODS

2.1 | Animals and samples

This observational cross-sectional study was conducted on dogs with a confirmed diagnosis of *B. canis* infection presented in March and April 2015 (the primary season for tick-borne disease) to a private veterinary practice in a suburban Belgrade (Serbia) municipality that is endemic for *B. canis*. Criteria for inclusion were: (1) acute onset of clinical signs consistent with *Babesia sp.* infection (24-48 hours of anorexia, fever, lethargy, pale or icteric mucous membranes and thrombocytopenia, leukopenia, and moderate anemia); (2) large *Babesia* organisms observed in thin blood smears stained with a Romanowsky stain (BioDiff, BioGnost, Zagreb, Croatia); (3) *B. canis*-positive polymerase chain reaction (PCR; Tick/Vector Comprehensive RealPCR Panel Canine, IDEXX Laboratories, Westbrook, Maine); and (4) negative serology for *Dirofilaria immitis*, *Ehrlichia* sp., *Anaplasma* sp., and *Borrelia* sp. (SNAP 4Dx Plus, IDEXX Laboratories). Dogs with visible wounds, neoplasia, and clinical signs or diagnoses of ectoparasites, allergies, and endocrinopathies were excluded. After blood collection and laboratory diagnostics, all dogs received a standard single SC dose of imidocarb-dipropionate (6.6 mg/kg

of body weight). All dogs were monitored for 15 days and the outcome (died or recovered) was recorded.

Control dogs were owned by staff members at the Faculty of Veterinary Medicine, University of Belgrade, Serbia. The dogs were determined to be clinically healthy based on physical examination and had no history of *B. canis* infection, negative results for *Babesia* by blood smear examination and PCR, and negative results for other organisms (SNAP 4Dx Plus, IDEXX Laboratories). Blood was collected as part of a health check before routine neutering.

Residual serum samples collected for routine laboratory diagnostic testing were used for all biochemical analyses. Blood samples were collected from the cephalic vein, placed in evacuated glass tubes without anticoagulant (Vacutainer, Becton Dickinson, Franklin Lakes, New Jersey) and centrifuged at 1500*g* for 10 minutes. Serum was harvested, placed in Eppendorf tubes, and stored at −20°C for up to 60 days until analysis.

All dog owners provided signed informed consent that the residual blood and serum samples obtained for diagnostic purposes could be used for a scientific study. The Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade, approved this study, and permission was obtained according to the Law for Animal Welfare from the Ministry of Agriculture and Environmental Protection, Republic of Serbia (permission number 323-07-03455/2015-05/3).

2.2 | Serum amyloid A as an indicator of the APR

Serum amyloid A concentration was determined using a solid sandwich ELISA that was previously validated for dogs (Tridelta Development Ltd, Maynooth, County Kildare, Ireland).[15] The intra-assay precision of the assay is <10% and the detection limit is 0.8 μg/mL. Absorbance was measured in microtiter plates on an ELISA plate reader (Elx800, Biotek Instruments, Highland Park, Winooski, Vermont) at 450 nm.

2.3 | Cholesterol, triglycerides, and phospholipid analysis

Serum was analyzed for total cholesterol and triglycerides, using routine clinical biochemistry kits according to the manufacturer's instructions (CHSL-0507 and TGML-0517, ELITech Clinical Systems, Sées, France) on a Technicon RA-XT automated biochemistry analyzer (Bayer Diagnostics, Swords, Co, Dublin, Ireland). Manufacturer controls were used (CONT 0060 and CONT0061 ELITech Clinical Systems). Total serum phospholipid concentration was determined by the method of Zilversmit and Davis.[16] Standard solution of KH_2PO_4 was prepared for use as a control (PO662, Sigma-Aldrich Chemie, Germany).

2.4 | Complete blood count and biochemistry analysis

Complete blood counts were done on an impedance-based hematology analyzer (Abacus Junior Vet, Diatron, Vienna, Austria) within 2 hours of sampling. Total protein, albumin, glucose, urea nitrogen and creatinine concentrations and alanine-aminotransferase (ALT), aspartate-aminotransferase (AST), and alkaline phosphatase (ALP) activities were measured using routine clinical biochemistry kits according to the manufacturer's instructions (Elitech, Puteaux, France) on a Technicon RA-XT automated biochemistry analyzer (Bayer).

2.5 | Serum lipoprotein electrophoresis

Serum lipoproteins were separated by commercial agarose gel electrophoresis and stained with Fat Red 7B according to manufacturer guidelines (SAS Lipoprotein, Helena Laboratories, Beaumont, Texas). Canine lipoproteins separate into the following bands based on migration distance in the gel: α1- (HDL-2), α2- (HDL-1), pre-β (very-low-density lipoproteins [VLDL]), and β (LDL). Because pre-β and β bands are difficult to distinguish in dogs, they were combined and reported as triglyceride-rich lipoproteins (TRL).[17]

Within-run (10 replicates of the same sample on a single gel) and between-run (a single sample run on 10 different gels) precision for relative lipoprotein concentrations was tested. Within-run and between-run precision for TRL was 6.5% and 12.6%, respectively; within-run and between-run precision for α-lipoproteins was 7.9% and 11.3%, respectively. Densitometry was performed by scanning the gels (Epson Perfection V800 Scanner, Seico Epson Corp., Indonesia) and calculating the relative lipoprotein concentration in each band as a percentage of the optical absorbance of that fraction (TotalLab TL120, Nonlinear Dynamics Ltd, Newcastle, UK). Agreement regarding the position of bands was determined by consensus of 3 of the authors (Z. Milanović, A. Ilić Božović, and M. Kovačević Filipović). Absolute concentrations of lipoproteins were not determined because according to the manufacturer (Helena Laboratories), the lipid stain has a greater affinity for triglycerides and cholesterol esters than for free cholesterol and phospholipids. The maximal migration distance of α-lipoprotein bands in control dogs was measured and the average value was used to normalize maximal migration distances for all samples, including controls.

2.6 | Lipoprotein morphology (diameter)

The diameter of HDL and TRL particles was determined by polyacrylamide gradient gel electrophoresis (PAGGE) with a 3%-31% gradient. Gels were prepared using a Hoefer SE 675 system (Amersham Pharmacia Biotech, Vienna, Austria) for vertical electrophoresis (Hoefer SE 600 Ruby system, Amersham Pharmacia Biotech). Electrophoresis time and voltage were 13 minutes at 60 V, followed by 20 minutes at 170 V, and 20 hours at 200 V. Gels were stained with Sudan Black B for lipids. Calibration curves for determining the diameter of HDL and TRL particles were prepared using carboxylated polystyrene microsphere beads (40 nm diameter) and proteins with high molecular weights: thyroglobulin (17.0 nm diameter), ferritin (12.2 nm), lactate dehydrogenase (8.4 nm), and albumin (7.1 nm). Gels were scanned with Image Scanner III (Amersham Pharmacia Biotech) and the Magic Scan software (version 4.6;1999; UMAX Data Systems, Inc, Fremont, California). Gels were analyzed using Image Quant software (version 5.2; 1999; Molecular Dynamics, Sunnyvale, California) as previously described.[18] Briefly, the diameter of the most prominent peak in the HDL region of each scan,

as determined by the software, was designated as the dominant HDL diameter (nm) and used for statistical analysis.[18]

2.7 | Apolipoprotein A-1 radioimmunoassay

Serum ApoA-1 concentration was determined using a radioimmunoassay as described previously, with minor modifications.[19] Polystyrene star tubes (Maxisorb Thermo Scientific, Denmark) were coated with ApoA-1 rabbit polyclonal antibody (0.5 μg/tube, sc-30089, Santa Cruz Biotechnology, Santa Cruz, California) in 0.05 M phosphate buffer, pH 7.2-7.4 with 0.15 M NaCl (PBS) (Merck, Germany) and incubated at 4°C overnight for antibody adsorption. After incubation, the tubes were rinsed with PBS and uncoated tube sites were blocked with 1% bovine serum albumin in PBS for 2 hours at 37°C. After blocking, tubes were again rinsed with PBS. Antibody radiolabeling with I^{125} was done by the method of Hunter and Greenwood[20] using 0.025 mg of ApoA-1 antibody and 0.5 mCi of I^{125} (Institute for Isotopes Co, Ltd, Budapest, Hungary). Canine serum samples were diluted (1:2) in PBS containing 0.5% casein sodium salt from bovine milk (Sigma-Aldrich Chemie). All samples (100 μL) were incubated with 100 μL of radiolabeled ApoA-1 antibodies (5×10^5 cpm/tube) overnight at room temperature (RT, 21°C-23°C) in antibody-coated tubes. After incubation, the tubes were rinsed, and bound radioactivity was measured in a Wizard 1470 Automatic γ-counter (PerkinElmer, Inc, Wellesley, Massachusetts). The concentration of ApoA-1 in canine serum samples was expressed in arbitrary units (U) obtained by normalizing the actual cpm/tube with the average cpm/tube of control samples.

2.8 | Statistical analysis

Data were analyzed using the MedCalc statistical software (version 16.2.1, Ostend, Belgium). Kolmogorov-Smirnov testing indicated the data were not normally distributed; therefore, differences between infected and control dogs were evaluated using the Mann-Whitney test and data reported as median and range (minimum-maximum). A P value of <.05 was considered significant. Spearman's rank correlation coefficients (rho) were determined to assess the correlation between SAA and ApoA-1 with HDL diameter and to assess the correlation between SAA and ApoA-1 levels.

3 | RESULTS

Twenty-nine dogs met the inclusion criteria for acute *B. canis* infection. The group consisted of 14 male and 15 female dogs of various breeds and with a median age of 3 (1–13) years. The clinical outcome for all of the dogs was full recovery (none of the dogs died). Control dogs consisted of 5 male and 5 female dogs with a median age of 3 (1–6) years. There was no significant difference in sex ratio or age between infected and control groups. Dogs with acute *B. canis* infection had been anorexic for 24-48 hours, with a median rectal temperature of 40.1°C (range, 39.1°C-41.2°C), tachycardia, and nonpalpable spleen; lymphadenomegaly was not observed.

Dogs with acute *B. canis* infection had significantly lower hematocrit, neutrophil, lymphocyte, and platelet counts compared with control dogs (Table 1). Based on reference limits used in our laboratory,

TABLE 1 Hematologic and serum biochemical results (median, minimum, and maximum values) in dogs with acute *Babesia canis* infection at the time of presentation, as compared with clinically healthy control dogs

Analyte	*B. canis*-infected (n = 29)	Control (n = 10)	Reference interval[a]	P[b]
RBC ($\times 10^{12}$/L)	5.6 (1.4-7.5)	6.7 (5.9-7.5)	5.5-8.5	.03
HGB (g/L)	135 (33-180)	163 (136-181)	120-180	.05
HCT (%)	35.0 (9.0-46.4)	45.2 (37.0-52.0)	37.0-55.0	.008
Total WBC ($\times 10^9$/L)	5.7 (2.3-32.8)	10.5 (6.4-13.9)	6.0-17.0	.01
NEUT ($\times 10^9$/L)	4.5 (1.2-27.4)	9.0 (3.6-11.5)	3.0-12.0	.02
LYM ($\times 10^9$/L)	0.99 (0.34-4.85)	3.60 (1.00-3.79)	1.0-4.8	.01
PLT ($\times 10^9$/L)	46 (20-556)	333 (233-433)	200-500	.001
Total protein (g/L)	60 (40-97)	55 (54-70)	54-75	NSD
Albumin (g/L)	29 (21-38)	29 (25-31)	23-31	NSD
Globulins (g/L)	38 (17-69)	22 (20-32)	16-36	NSD
Glucose (mmol/L)	5.2 (3.2-8.4)	5.2 (4.5-6.1)	4.2-6.6	NSD
ALT (U/L)	37.5 (13-264)	45.5 (30-61)	10-109	NSD
AST (U/L)	48 (13-197)	18 (14-39)	13-60	.02
ALP (U/L)	163 (17-871)	54 (34-77)	11-114	.008
Creatinine (μmol/L)	129.0 (73.1-176.7)	92.4 (78.7-129.4)	54.0-150.0	.04
Urea (mmol/L)	7.3 (3.6-13.0)	5.7 (3.8-7.2)	2.9-10.0	.007

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCT, hematocrit; HGB, hemoglobin; LYM, lymphocytes; NEUT, segmented neutrophils; NSD, not significantly different; PLT, platelets; RBC, red blood cells; WBC, white blood cells.
[a]Reference intervals are from the clinical laboratory in the Faculty of Veterinary Medicine, University of Belgrade, Serbia.
[b]Significant differences (P < .05) between infected and control groups (Mann-Whitney test).

dogs with *B. canis* infection had anemia (18/29), leukopenia (17/29), leukocytosis (1/29), lymphopenia (16/29), lymphocytosis (1/29), thrombocytopenia (26/29), and thrombocytosis (1/29). Infected dogs also had significantly higher serum AST and ALP activities and creatinine and urea concentrations compared with control dogs (Table 1). Dogs with *B. canis* infection had hyperproteinemia (5/29), hypoproteinemia (10/29), hyperalbuminemia (13/29), hypoalbuminemia (4/29), hyperglobulinemia (8/29), hyperglycemia (4/29), hypoglycemia (8/29), azotemia (6/29), and increased ALT (2/29), AST (12/29), and ALP (20/29) activities.

All dogs with *B. canis* infection had a strong APR based on a significant increase in SAA concentration (median, 168.3 μg/mL; range, 98.1-716.2 μg/mL) compared with that of control dogs (3.2 μg/mL, 2.0-4.2 μg/mL; $P < .001$) (Figure 1).

Dogs with *B. canis* infection had significantly lower concentrations of total cholesterol and phospholipids compared with control dogs, whereas triglyceride concentration did not differ (Figure 2). Control dogs had clearly distinguishable lipoprotein bands for TRL, α2-lipoproteins (HDL-1), and α1-lipoproteins (HDL-2) (Figure 3A). Twenty of 29 (69%) dogs with *B. canis* infection lacked a separate HDL-2 band, with indistinct separation of α2- and α1-lipoproteins. The maximal migration distance of α-lipoprotein bands was shorter in dogs infected with *B. canis* (median, 0.90; range, 0.82-1.02), compared with control dogs (median, 1.02; range, 0.94-1.02; $P = .002$) (Figure 3B). Dogs with *B. canis* infection had lower relative concentrations of HDL compared with healthy controls, but relative TRL concentrations did not differ (Figure 3C).

Triglyceride-rich lipoprotein and HDL regions on PAGGE were separated by narrow distinct band(s) previously identified as α2-macroglobulin[21] (Figure 4A). Dogs with *B. canis* infection had an accumulation of larger HDL particles in the region between thyroglobulin (17 nm) and ferritin (12.2 nm) (Figure 4A). The dominant TRL diameter did not differ between infected and control dogs, whereas the dominant HDL diameter was significantly larger in dogs with *B. canis* infection (Figure 4B). Apolipoprotein A-1 concentration was significantly higher in dogs with *B. canis* as compared with control dogs (Figure 5). The dominant HDL diameter was positively correlated with SAA concentration (rho = .43; $P = .03$), but not with ApoA-1 concentration (rho = .36; $P = .06$). There was a significant positive correlation between SAA and ApoA-1 concentrations (rho = .63, $P < .001$).

4 | DISCUSSION

Patterns of lipids and lipoproteins in dogs with acute *B. canis* infection differed significantly from those in clinically healthy dogs. Decreased cholesterol and phospholipid concentrations, loss of HDL-2, and an increased dominant diameter of HDL particles were consistent with APR-induced changes in lipid metabolism and lipoprotein morphology, as described in humans and rodents. These findings support decreased reverse cholesterol transport as well as altered HDL morphology, possibly because of enrichment with SAA. Unlike in other species, ApoA-1 concentrations were increased in dogs with *B. canis*-induced APR. Further, triglyceride and TRL concentrations were unaffected, in contrast to previous findings in dogs with *B. canis* infection. These results confirm and expand upon our understanding of altered lipid metabolism in dogs with *B. canis*-induced APR, which might serve as a model for lipid alterations in dogs with acute inflammation.

High-density lipoprotein is a major carrier of free and esterified cholesterol and is also rich in phospholipids in healthy dogs,[7] such that the lack of HDL-2 in dogs with *B. canis* infection likely resulted in the lower serum total cholesterol and phospholipid concentrations in the present study. Low concentration of HDL-cholesterol occurs in dogs with babesiosis,[12,22] but the specific HDL type was not determined. Cattle with acute *B. bovis* infection have decreased total cholesterol concentration and a relative decrease in α-lipoproteins (HDL).[23] People infected with *Babesia* also have decreased HDL concentrations.[24]

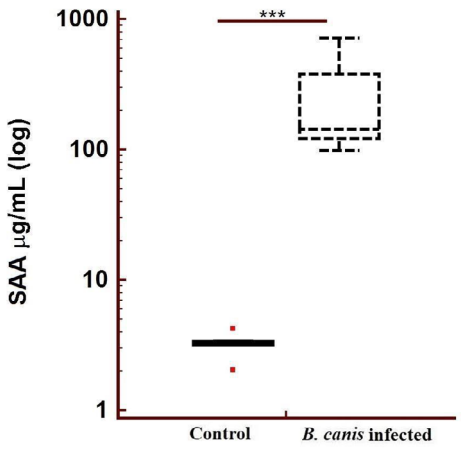

FIGURE 1 Serum amyloid A (SAA) concentration in dogs with acute *Babesia canis* infection (n = 29) indicate a major acute phase response, as compared with control dogs (n = 10). Boxes indicate the lower to upper quartile (25th-75th percentile) and median value. Whiskers extend to minimum and maximum values, with outliers shown as individual points (***$P < .001$)

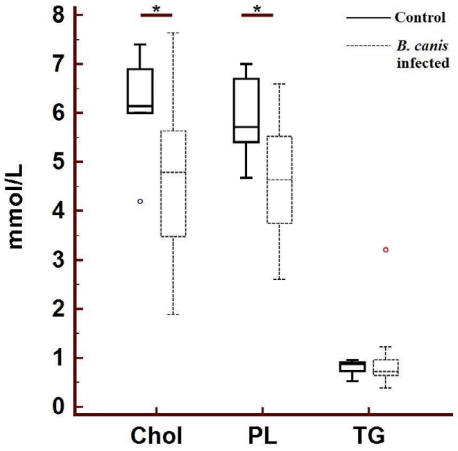

FIGURE 2 Concentrations of serum total cholesterol (Chol), phospholipids (PL), and triglycerides (TG) in dogs with *Babesia canis* infection and an acute phase response (n = 29) and in control dogs (n = 10). Boxes indicate the lower to upper quartile (25th-75th percentile) and median value. Whiskers extend to minimum and maximum values, with outliers shown as individual points (*$P = .02$ for Chol, *$P = .02$ for PL)

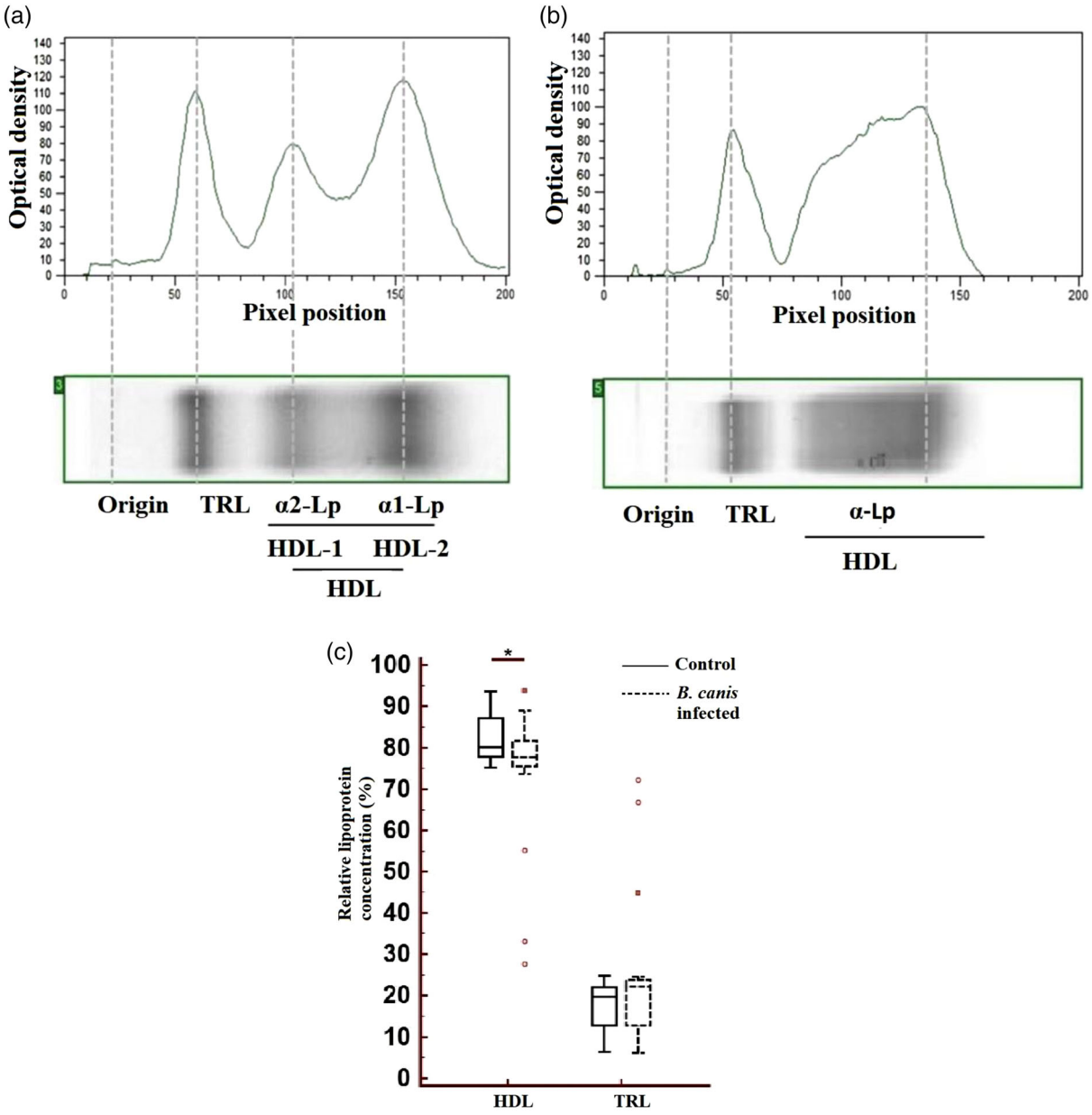

FIGURE 3 Representative lipoprotein (Lp) electrophoretic patterns on agarose gel for A, a control dog and B, a dog with *Babesia canis* infection, and C, the relative concentrations of triglyceride-rich lipoproteins (TRL) and high-density lipoproteins (HDL) in infected and control dogs. A, Control dogs have distinct peaks for TRL, HDL-1, and HDL-2. B, Dogs with *B. canis* infection and an acute phase response have indistinct separation between HDL-1 and HDL-2 and an overall shorter migration distance for all lipoproteins. Note the shorter migration distance of lipoproteins (150th notch on the scale) in the infected dog as compared with the clinically healthy dog (200th notch on the scale). C, Dogs with *B. canis* infection have a significantly lower relative HDL concentration compared with control dogs (*P = .04). Boxes indicate the lower to upper quartile (25th-75th percentile) and median value. Whiskers extend to minimum and maximum values, with outliers shown as individual points

Our results confirmed that HDL-2 was decreased concurrent with high SAA levels. One potential mechanism of decreased HDL in acute inflammation is increased endothelial lipoprotein lipase expression, which increases the hydrolysis of phospholipids and, in humans, leads to the catabolism and subsequent decrease of HDL in an APR.[25,26] Canine babesiosis increases cortisol and insulin concentration and decreases thyroid hormone concentration.[27,28] Theoretically, these changes would be expected to induce hypercholesterolemia. However, low normal cholesterol concentration was observed in dogs with *B. canis* infection in the present study, suggesting that increased utilization of cholesterol in cortisol synthesis may have contributed at least partly to lower cholesterol and HDL values.

The loss of distinctly separate HDL-1 and HDL-2 bands in lipoprotein electrophoresis in dogs with *B. canis*-induced APR was consistent with the alteration of HDL composition and an increase in the dominant HDL diameter, as assessed by PAGGE. Increased HDL diameter has been demonstrated repeatedly in vitro and in vivo in humans and mice with an APR, always in association with increases in SAA concentration.[5,6,29,30]

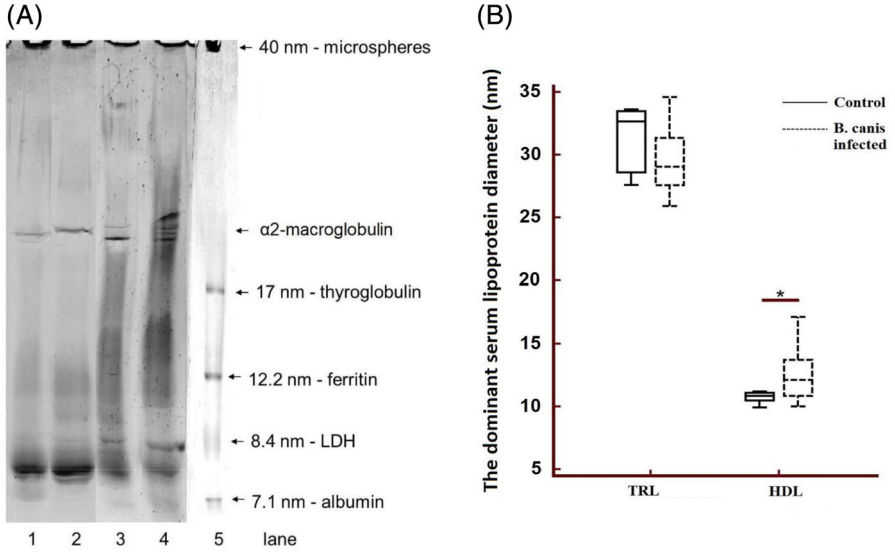

FIGURE 4 Diameter of triglyceride-rich lipoproteins (TRL) and high-density liproproteins (HDL) on polyacrylamide gradient gel electrophoresis (PAGGE). A, Polyacrylamide gradient gel electrophoresis stained with Sudan Black B for lipids. Control dogs (lanes 1 and 2), dogs with *Babesia canis* infection (lanes 3 and 4), and ladder proteins (lane 5; LDH indicates lactate dehydrogenase). Note that *B. canis*-infected dogs have larger lipoproteins in the HDL region between thyroglobulin and ferritin; also note the difference between control and infected dogs in the narrow distinct band(s) that represent α2-macroglobulin. B, Dogs with *B. canis* infection have significantly larger HDL diameter compared with control dogs (**P* = .04), with no difference in TRL diameter. Boxes indicate the lower to upper quartile (25th-75th percentile) and median value. Whiskers extend to minimum and maximum values, with outliers shown as individual points

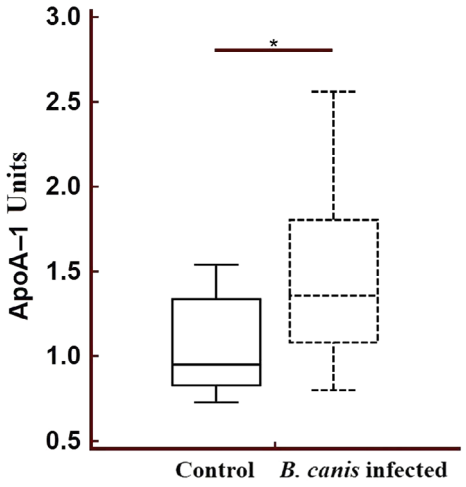

FIGURE 5 Relative apolipoprotein A-1 (ApoA-1) concentrations in dogs with *Babesia canis* infection and in control dogs (**P* = .02). Boxes indicate the lower to upper quartile (25th-75th percentile) and median value. Whiskers extend to minimum and maximum values

Indeed, we demonstrated a modest but significant positive correlation between the concentration of SAA and HDL diameter in the dogs with *B. canis*-induced APR. During an APR in bovines other lipophilic serum proteins are also bound to HDL particles.[31] In particular, hemolysis, the major cause of anemia in *B. canis* infection, can influence morphology of HDL through the binding of free hemoglobin and haptoglobin.[32]

Another potential contribution to expansion of HDL diameter in dogs with an APR is the lack of adequate HDL remodeling in the liver. In dogs, 60% of HDL cholesteryl esters are removed from the plasma through selective uptake by the liver via SR-B1.[33] In humans and mice with APRs, both hepatic lipase and SR-B1 are downregulated.[34] Based on investigations in humans using nuclear magnetic resonance, an APR results in fewer small- and medium-sized HDL particles, with no change (and hence a relative increase) in large HDL particles.[35] Thus, the decrease in smaller HDL-2 particles in dogs with *B. canis*-induced APR may have resulted from decreased HDL-2 formation because of altered hepatic lipase and SR-B1 function and/or increased HDL-2 catabolism by endothelial lipase.

In primates and rodents, decreased synthesis or increased catabolism of ApoA-1 in association with increased SAA synthesis has led to the conclusion by others that the 2 molecules are reciprocally regulated by inflammation.[3,36] Unexpectedly, dogs with *B. canis*-induced APR and loss of HDL-2 in our study had higher relative ApoA-1 concentrations than control dogs. These results confirm those in a recent study of dogs with babesiosis (in which ApoA-1 was measured using mass spectrometry),[13] but contrast with those in most other species and types of inflammation, including dogs with *Leishmania* infection[37] and children with malaria (*Plasmodium falciparum*),[38] in which ApoA-1 values decrease together with decreased cholesterol values. in vitro studies show that binding of SAA to HDL leads to physical displacement of ApoA-1 from HDL particles.[5,39] Nevertheless, expression of SAA in the absence of inflammation does not decrease ApoA-1 levels in transgenic mice.[40] People homozygous for CETP mutations have increased ApoA-1 levels, but in conjunction with increased HDL cholesterol.[41] Thus, the mechanism of increased ApoA-1 in dogs with *B. canis* remains uncertain and may be species- or disease-specific. Increased ApoA-1 in dogs with *B. canis* infection also might be the

consequence of its increased synthesis or decreased catabolism by the kidneys.[42] Necrosis of renal tubular epithelial cells occurs in fatal cases of *B. canis* infection,[43] but all infected dogs in the present study recovered. Additional studies of altered renal catabolism and other potential mechanisms of increased ApoA-1 in dogs with *B. canis* infection and the APR are warranted.

In dogs with *B. canis*-induced APR in this study, serum concentrations of triglycerides and TRL did not differ from those in control dogs. In contrast, humans and rodents administered lipopolysaccharide (LPS) develop hypertriglyceridemia as a result of increased production (with low-dose LPS) or decreased clearance (with high-dose LPS) of VLDL particles.[44] In our study, dogs were anorexic for at least 24 to 48 hours before presentation, which may have lowered triglyceride and TRL levels. Short-term fasting (36 hours) induces a decrease in TRL in healthy dogs,[45] raising the possibility that any increase in VLDLs caused by inflammation might have been masked, as has been described for primates and rodents.[1] In previous studies, serum triglycerides concentration in dogs was increased after the treatment of *B. canis* infection, suggesting the involvement of this class of lipids in the APR.[12] This reaction is proposed to be beneficial as triglycerides are a source of fatty acids and a part of the innate defense system that neutralizes lipophilic toxins.[1]

5 | LIMITATIONS

Although all the dogs had a noncomplicated form of *B. canis* infection without autoimmune hemolytic anemia, acute renal failure, and septic shock and marked APR, natural infections are heterogeneous, and the variable amount of time between infection and presentation might have differentially affected lipoprotein changes in infected dogs. Because all dogs survived and recovered, we were unable to test the association between severity of disease or prognosis and lipid and lipoprotein variables. Also, although concurrent vector-borne infections were excluded using the SNAP 4Dx Plus test, we cannot rule out the presence of infectious agents for which testing was not done. Potential comorbidities such as chronic kidney disease also were not ruled out and could have influenced the results. Although a relatively small number of dogs were tested in this study, the population comprised all dogs meeting the inclusion criteria within the designated *B. canis* tick season.

6 | CONCLUSION

Major changes associated with *B. canis*-induced APR in dogs are related to concentration, composition, and morphology of HDL particles pointing to an altered reverse cholesterol transport. Parallel ApoA-1 and SAA concentration increase is a unique still unexplained pathophysiological finding that warrants further assessment of the prognostic significance.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

The Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade, approved this study, and permission was obtained according to the Law for Animal Welfare from the Ministry of Agriculture and Environmental Protection, Republic of Serbia (permission number 323-07-03455/2015-05/3).

HUMAN ETHICS APPROVAL DECLARATION

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

ORCID

Zorana Milanović https://orcid.org/0000-0001-9953-3269

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