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

Zorana Milanović1 | Jelena Vekić2 | Vladimir Radonjić3 | Anja Ilić Božović1 | Aleksandra Zeljković2 | Jelena Janac2 | Vesna Spasojević-Kalimanovska2 | Jesse Buch4 | Ramaswamy Chandrashekar4 | Žanka Bojić-Trbojević5 | Ljiljana Hajduković5 | Mary M. Christopher6 | Milica Kovačević Filipović1

1Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Serbia
2Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia
3Small animal practice "Petrovac", Beograd, Serbia
4IDEXX Laboratories, Westbrook, Maine
5INEP - Institute for the Application of Nuclear Energy, University of Belgrade, Zemun, Serbia
6Department 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

Funding information
IDEXX Laboratories, Inc., Westbrook, Maine, USA; LKB Vertriebs Ges.m.b.h., Belgrade; Ministarstvo Prosvete, Nauke i Tehniolškog Razvoja, Grant/Award Numbers: 175035, 175061

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.

© 2019 The Authors. Journal of Veterinary Internal Medicine published by Wiley Periodicals, Inc. on behalf of the American College of Veterinary Internal Medicine.
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. Some changes, such as a decrease in high-density lipoprotein (HDL)-cholesterol, are related to poor prognosis and death in septic humans and rodents.

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. 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). ApoA-1 is indispensable for cholesterol efflux and esterification, and for the remodeling of HDL particles. Furthermore, in acute inflammation, HDL becomes enriched with SAA, resulting in further modification of its morphology and diameter. The overall consequence of an APR is decreased cholesterol efflux, altered HDL morphology, and decreased cholesterol uptake through SR-B1 in the liver.

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. Canine HDL particles form distinct α1 and α2 bands in electrophoretic gels that correspond quantitatively to HDL-2 and HDL-1. Dogs lack cholesterol ester transfer protein (CETP) activity, which in humans is involved in transferring cholesteryl esters from HDL to low-density lipoproteins (LDLs). 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. Total cholesterol and HDL-cholesterol levels are lower at the time of presentation than after treatment with imidocarb-dipropionate, and ApoA-1 concentration is higher in dogs with acute babesiosis. However, in those studies, the method used for HDL cholesterol was not recommended for use with canine serum, 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). 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 (Eix800, 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, Sèves, 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. Standard solution of KH₂PO₄ 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).

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 Bozović, 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. 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 I125 was done by the method of Hunter and Greenwood20 using 0.025 mg of ApoA-1 antibody and 0.5 mCi of I125 (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 x 10⁵ 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 intervala | Pb |
|-----------------------|------------------------------|------------------|---------------------|----|
| RBC (×10¹²/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 (×10⁹/L)    | 5.7 (2.3-32.8)               | 10.5 (6.4-13.9)  | 6.0-17.0            | .01|
| NEUT (×10⁹/L)         | 4.5 (1.2-27.4)               | 9.0 (3.6-11.5)   | 3.0-12.0            | .02|
| LYM (×10⁹/L)          | 0.99 (0.34-4.85)             | 3.60 (1.00-3.79) | 1.0-4.8             | .01|
| PLT (×10¹²/L)         | 46 (20-556)                  | 333 (233-433)    | 200-500             | .001|
| Total protein (g/L)   | 60 (40-97)                   | 55 (54-70)       | 54-75               | MSD|
| Albumin (g/L)         | 29 (21-38)                   | 29 (25-31)       | 23-31               | MSD|
| Globulins (g/L)       | 38 (17-69)                   | 22 (20-32)       | 16-36               | MSD|
| Glucose (mmol/L)      | 5.2 (3.2-8.4)                | 5.2 (4.5-6.1)    | 4.2-6.6             | MSD|
| ALT (U/L)             | 37.5 (13-264)                | 45.5 (30-61)     | 10-109              | MSD|
| 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.

aReference intervals are from the clinical laboratory in the Faculty of Veterinary Medicine, University of Belgrade, Serbia.

bSignificant 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), hyperglobulinemia (8/29), hypoglycemia (4/29), 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; median, 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 (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, \( P < .001 \)).

### 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, 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, 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). People infected with Babesia also have decreased HDL concentrations.
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 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.
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. 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. 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. In humans and mice with APRs, both hepatic lipase and SR-B1 are downregulated. 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. 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. 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), but contrast with those in most other species and types of inflammation, including dogs with *Leishmania* infection and children with malaria (*Plasmodium falciparum*), 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. However, expression of SAA in the absence of inflammation does not decrease ApoA-1 levels in transgenic mice. People homozygous for CETP mutations have increased ApoA-1 levels, but in conjunction with increased HDL cholesterol. 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.\textsuperscript{42} Necrosis of renal tubular epithelial cells occurs in fatal cases of \textit{B. canis} infection,\textsuperscript{13} 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 \textit{B. canis} infection and the APR are warranted.

In dogs with \textit{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 hypertriglycerideremia as a result of increased production (with low-dose LPS) or decreased clearance (with high-dose LPS) of VLDL particles.\textsuperscript{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,\textsuperscript{45} raising the possibility that any increase in VLDLs caused by inflammation might have been masked, as has been described for primates and rodents.\textsuperscript{3} In previous studies, serum triglycerides concentration in dogs was increased after the treatment of \textit{B. canis} infection, suggesting the involvement of this class of lipids in the APR.\textsuperscript{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.\textsuperscript{1}

\section{LIMITATIONS}

Although all the dogs had a noncomplicated form of \textit{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 \textit{B. canis} tick season.

\section{CONCLUSION}

Major changes associated with \textit{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.

\section{ACKNOWLEDGMENTS}

This paper was presented as poster presentation at ESVCP-ESVONC Congress 2016, Nantes, France. This work was supported by a grant from the Ministry of Education and Science, Republic of Serbia (Project No. 175061). The work performed in laboratories at the Department of Medical Biochemistry, Faculty of Pharmacy, was financially supported by a grant from the Ministry of Education, Science and Technological Development, Republic of Serbia (Project No. 175035). Tick/Vector Comprehensive RealPCR Panel Canine and complete IFAT titer for \textit{B. canis}, \textit{B. vogeli}, \textit{B. gibsoni} were funded by IDEXX Laboratories, Inc (Westbrook, Maine), Branch of LKB Vertriebs Ges.m.b.h., Belgrade, kindly provided ApoA-1 antibody used in this study. Special gratitude is due to Nebojša Dovezenski for assistance with electrophoretic techniques.

\section{CONFLICT OF INTEREST DECLARATION}

Authors declare no conflict of interest.

\section{OFF-LABEL ANTIMICROBIAL DECLARATION}

Authors declare no off-label use of antimicrobials.

\section{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).

\section{HUMAN ETHICS APPROVAL DECLARATION}

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

\section{ORCID}

Zorana Milanović \(\text{https://orcid.org/0000-0001-9953-3269}\)

\section{REFERENCES}

1. Khovdiphukit W, Kim MS, Memon RA, et al. Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. \textit{J Lipid Res.} 2004;45:1169-1196.
2. Morin EE, Guo L, Schwendeman A, et al. HL in sepsis – risk factor and therapeutic approach. \textit{Front Pharmacol.} 2015;6:244.
3. Cabana VG, Lukens JR, Rice KS, Hawkins TJ, Getz GS. HDL content and composition in acute phase response in 3 species: triglyceride enrichment of HDL a factor in its decrease. \textit{J Lipid Res.} 1996;37:2662-2674.
4. Mineo C, Shaul PW. Regulation of signal transduction by HDL. \textit{J Lipid Res.} 2013;54:2315-2324.
5. Coetzees GA, Strachang AF, Van Der Westhuynzen DR, et al. Serum amyloid A-containing human high density lipoprotein 3 density, size, and apolipoprotein composition. \textit{J Biol Chem.} 1986;261:9644-9651.
6. Lindhorst E, Young D, Bagshaw W, Hyland M, Kiselevsky R. Acute inflammation, acute phase serum amyloid A and cholesterol metabolism in the mouse. \textit{Biochim Biophys Acta.} 1997;1339:143-154.
7. Malondano EN, Romero JR, Ochoa B, Aveldaño MI. Lipid and fatty acid composition of canine lipoproteins. \textit{Comp Biochem Physiol B Biochem Mol Biol.} 2001;128:719-729.
13. Kuleš J, Barić Rajaf R, et al. Canine babesiosis: where do we stand? Acta Vet-Bulg. 2018;68:127-160.
14. Mrljak V, Kučer N, Kuleš J, et al. Serum concentrations of eicosanoids and lipids in dogs naturally infected with Babesia canis. Vet Parasitol. 2014;201:24-30.
15. Martinez-Subiela S, Ceron JJ. Validation of commercial assays for high density lipoprotein cholesterol levels and cholesteryl ester transfer protein activity in six species of healthy experimental animals. Biol Pharm Bull. 2001;24:579-581.
16. Šilpić P, Kuleš J, Barić Rajaf R, et al. Canine babesiosis: where do we stand? Acta Vet-Bulg. 2018;68:127-160.
17. Kawasumi K, Kashiwado N, Okada Y, et al. Age effects on plasma haptoglobin, c-reactive protein and serum amyloid A in dogs. Arch Med. Vet. 2005;37:61-66.
18. Zylwersmit DB, Davis AK. Microdetermination of plasma phospholipids by trichloroacetic acid precipitation. J Lab Clin Med. 1950;35:155-160.
19. Kawasumi K, Kashiwado N, Okada Y, et al. Age effects on plasma cholesterol and triglyceride profiles and metabolite concentration in dogs. BMC Vet Res. 2014;10:57.
20. Vekic J, Topic A, Željkovic A, Jelic-Ivanovic Z, Spasojevic-Kalimanovska V. LDL and HDL subclasses and their relationship with Framingham risk score in middle-aged Serbian population. Clin Biochem. 2007;40:310-316.
21. Gojic-Bojic D, Bojic-Trbojevic ZT. Human carcinoma-associated and salivary mucins detected by anti-bovine submaxillary mucin antibodies. Biochemistry (Moscow). 2006;71:9-17.
22. Hunter WM, Greenwood FC. Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature. 1962;194:495-496.
23. Rainwater DL, Moore PH, Gamboa IO. Improved method for making nondenaturing composite gradient gels for the electrophoretic separation of lipoproteins. J Lipid Res. 2004;45:773-775.
24. Rossi G, Kuleš J, Rafaj RB, et al. Relationship between paraoxonase 1 activity and high density lipoprotein concentration during naturally occurring babesiosis in dogs. Res Vet Sci. 2014;97:318-324.
25. Goodger BV, Wright LG, Mahoney DF. Babesia bovis (Argentina): studies of plasma lipids and lipoproteins during acute infections in cattle. Parasit Eng. Parasitol. Res. 1981;65:271-276.
26. Cunha BA, Crean J. Lipid abnormalities in babesiosis. Am J Med. 2000;108:758-759.
27. McCoy MG, Sun GS, Marchadier D, Maugées C, Glick JM, Rader DJ. Characterization of the lipolytic activity of endothelial lipase. J Lipid Res. 2002;43:921-929.
28. Badellino KO, Wolfe ML, Reilly MP, et al. Endothelial lipase concentrations are increased in metabolic syndrome and associated with coronary atherosclerosis. PLoS Med. 2006;3:e22.
29. Matijačko V, Torti M, et al. Serum cortisol and insulin concentrations in dogs naturally infected with Babesia canis. Vet Archiv. 2014;84:551-562.
30. Zygner W, Gojska-Zygner O, et al. Low T3 syndrome in canine babesiosis associated with increased serum IL-6 concentration and azotaemia. Vet Parasitol. 2015;211:23-27.
31. Clifton PM, Mackinnon AM, Barter PJ. Effects of serum amyloid A protein (SAA) on composition, size, and density of high density lipoproteins in subjects with myocardial infarction. J Lipid Res. 1985;26:1389-1398.
32. Abo-Dohmae S, Kato KH, Kumon Y, et al. Serum amyloid A generates high density lipoprotein with cellular lipid in an ABCA1- or ABCA7-dependent manner. J Lipid Res. 2006;47:1542-1550.
33. Novakovic F, Filipovic M, O'Reilly EL, Eckersall PD. Bovine LDL and HDL proteome in health and inflammatory state. In: de Almeida A, Cecilli F, Eckersall D, et al., eds. Farm Animal Proteomics 2014: Proceedings of the 5th Management Committee Meeting and 4th Meeting of Working Groups 1, 2 & 3 of Cost Action FA 1002; November 17-18, 2014; Milano, Italy. The Netherlands: Wageningen Academic Publishers; 2014:29-32.
34. Palić Š, Grijalva V, Hama S, et al. Hemoglobin and its scavenger protein haptoglobin associate with apol-1-containing particles and influence the inflammatory properties and function of high density lipoprotein. J Biol Chem. 2009;284:18292-18301.
35. Ouguerram K, Nguyen P, Krempf M, et al. Selective uptake of high density lipoprotein cholesteryl ester in the dog, a species lacking in cholesteryl ester transfer protein activity. Comp Biochem Physiol Part B Biochem Mol Biol. 2004;138:339-345.
36. Feingold KR, Memon RA, Moser AH, Shigenaga JK, Grunfeld C. Endotoxin and interleukin-1 decrease hepatic lipase mRNA levels. Arteriosclerosis. 1999;14:379-387.
37. de la Llera Moya M, McGillicuddy FC, Hinkle CC, et al. Inflammation modulates human HDL composition and function in vivo. Arteriosclerosis. 2012;22:290-394.
38. Han CY, Chiba T, Campbell JS, et al. Reciprocal and coordinate regulation of serum amyloid A versus apolipoprotein A-1 and paraoxonase-1 by inflammation in murine hepatocytes. Arterioscler Thromb Vasc Biol. 2006;26:1806-1813.
39. Escribano D, Tvarijonaviciute A, Kocaturk M, et al. Serum apolipoprotein-A1 as a possible biomarker for monitoring treatment of canine leishmaniosis. Comp Immunol Microbiol Infect Dis. 2016;49:82-87.
40. Orimadegun AE, Orimadegun BE. Serum apolipoprotein-A1 and cholesterol levels in Nigerian children with Plasmodium falciparum infection. Med Princ Pract. 2015;24:318-324.
41. Mišić T, Yamada T, Yamadera T, Ozaki K, Inano K, Okada M. Serum amyloid A protein generates preβ1 high-density lipoprotein from α-migrating high-density lipoprotein. Biochemistry. 1999;38:16958-16962.
42. Hosoi H, Webb NR, Glick JM, et al. Expression of serum amyloid A protein in the absence of the acute phase response does not reduce HDL cholesterol or apol-1 levels in human apoA-I transgenic mice. J Lipid Res. 1999;40:648-653.
43. Arai T, Tsukada T, Murase T, Matsumoto K. Particle size analysis of high density lipoproteins in patients with cholesteryl ester transfer protein deficiency. Clin Chim Acta. 2000;301:103-117.
44. Yang H, Fogo AB, Kon V. Kidneys: key modulators of high-density lipoprotein levels and function. Curr Opin Nephrol Hypertens. 2016;25:174-179.
45. Måthé A, Dobos-Kovács M, Vörös K, Histological K. Ultrastructural studies of renal lesions in Babesia canis infected dogs treated with imidocarb. Acta Vet Hung. 2007;55:511-523.
46. Feingold KR, Hardardottir I, Memon R, et al. Effect of endotoxin on cholesterol biosynthesis and distribution in serum lipoproteins in Syrian hamsters. J Lipid Res. 1993;34:2147-2158.
47. Dedić-Delić A, Milanović Z, Bozović-Ilić A, et al. Effects of short-term fasting on lipid and lipoprotein concentrations in healthy lean dogs. Vet Glaz. 2018;72:35-43.

How to cite this article: Milanović Z, Vekić J, Radojičić V, et al. Association of acute Babesia canis infection and serum lipid, lipoprotein, and apoprotein concentrations in dogs. J Vet Intern Med. 2019;33:1686–1694. https://doi.org/10.1111/jvim.15537