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## Identification of serum proteins in dogs naturally infected with *Anaplasma phagocytophilum* and *Borrelia burgdorferi*: a pilot study

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**ABSTRACT:** Serum proteomic analysis would aid in better understanding the pathophysiology of several diseases. The aim of this study was to identify the serum proteomes of dogs with anaplasmosis and Lyme disease using a proteomic approach. Diseases were diagnosed by a commercial rapid in-clinic ELISA. *Borrelia* antibodies were evaluated by IFAT. Four groups were designated: symptomatic dogs with anaplasmosis (n=5), dogs with Lyme disease (n=5), dual-positive dogs (n=5), and healthy control dogs (n=5). Serum samples were collected before treatment. Two-dimensional electrophoresis of pooled samples in each group were run in triplicate. Ten out of 57 differentially expressed spots between groups were evaluated for identification by mass spectrometry.

Compared to those of controls, levels of vitamin D-binding protein (VDBP), glycoprotein-9 (GP9) and kininogen-1 (KGN-1) decreased, while haptoglobin (Hp) and immunoglobulin (Ig) heavy chain levels increased in dual infection group. Serum apolipoprotein-A1 (Apo-A1) levels decreased in dogs with anaplasmosis, Lyme disease and dual infections compared to those in control dogs. Serum clusterin levels decreased in dogs with anaplasmosis but were not differentially expressed in dogs with Lyme disease or dogs with dual infections compared to those in control dogs. Calpain-3 decreased in dogs with anaplasmosis and Lyme disease.

This study showed that many protein levels might be changed in dogs with naturally acquired anaplasmosis and Lyme disease. Understanding the role of these proteins in different biological processes can provide information of interest for diagnostic and therapeutic approaches for these clinical conditions.

**Keywords:** Anaplasmosis, Lyme disease, proteomic, dog

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## INTRODUCTION

*Anaplasma phagocytophilum* (*A. phagocytophilum*) and *Borrelia burgdorferi* (*B. burgdorferi* s.l.) are two tick-borne diseases that affect humans and animals in Africa, the Middle East, Europe and North America. *A. phagocytophilum* is a gram-negative obligate organism that is the causative agent of anaplasmosis in dogs, humans, cats and horses as well as tick-borne fever in ruminants. *B. burgdorferi* s.l. causes Lyme disease, also called borreliosis, which is the most frequently diagnosed vector-borne disease (VBD) in humans and dogs (Kybicova et al, 2009; Mc Causland et al, 2011; Hovius, 2015).

Coinfections are common, with these VBDs transferred by the same ticks to mammals and showing clinical symptoms within 20 days to 3 months after getting bitten by an infected tick. Increased body temperature, lethargy, lameness, and painful joints are frequently observed symptoms. Diagnosis of Lyme disease in daily practice can be challenging (Hovius, 2005); however, Lyme arthritis is seen on the extremity close to the tick bite area (Ettinger and Feldman, 2010). Hepatomegaly and increased serum liver enzyme levels can be seen with exposure to both organisms. In cases of anaplasmosis, pale mucous membranes and bleeding disorders due to thrombocytopenia, renal damage accompanied by proteinuria, haematuria, azotaemia (Greene, 2012), neurologic and cardiologic abnormalities (hyperesthesia, posterior paresis, epilepsy, meningoencephalitis, arrhythmia and cardiomyopathy) can also be seen (Agudelo et al, 2011; Janus et al., 2014; Kybicove et al, 2009; Schanilec et al 2010). Although serum analysis of specific antibodies (immunoglobulins) and PCR tests are more suitable for reliable diagnosis of Lyme disease and anaplasmosis, ELISA-based in-clinic rapid tests are commonly used as a diagnostic test with high specificity and sensitivity for both diseases (Stillman et al, 2014).

Screening of the disease in detail gives rise to an understanding of the complex pathophysiological mechanism in dogs and humans. Proteomic analysis can identify biomarkers that elucidate the pathophysiological changes associated with the infection and may have implications for better knowledge of the pathology of these VBDs. To the authors' knowledge, there are no data of serum proteomes on these VBDs and modifications in canine expressional proteomics of serum samples. Thus, the aim of this study was to investigate potential serum proteomes (bioindicators)

in dogs with anaplasmosis, Lyme disease and dual infections from both pathogens by using a proteomic approach.

## MATERIALS AND METHODS

### Animals and sampling

For this study, 4 groups were created; dogs with anaplasmosis (Group-1), dogs with Lyme disease (Group-2) and dogs with dual infection (Group-3), and healthy control dogs (Group-4). These groups consisted of a total of 20 dogs (5 dogs in each) with 6 different breeds (Golden Retrievers, Terriers, Boxers, German Shepherds, Cavalier King Charles Spaniels and mixed breed dogs). The mean age was  $6.2 \pm 2.4$  yrs in Group-1,  $8.4 \pm 3.2$  yrs in Group-2,  $7.5 \pm 2.6$  yrs in Group-3 and  $6.5 \pm 2.0$  yrs in Group-4.

The symptoms that were observed in our study population, which were compatible with a vector-borne disease were inappetence (2/Group-1, 3/Group-2, and 2/Group-3), lethargy (1/ Group-1, 2/ Group-2, and 1/ Group-3), fever (3/Group-1, 2/Group-2, and 1/Group-3), joint swelling (3/Group-2, 2/Group-3), and/or thrombocytopenia (5/Group-1, 4/Group-2, and 5/Group-3) and serum samples of the patients were collected for further diagnostic and evaluation steps. Diagnosis was based on a seropositive test result of a commercial rapid in-clinic ELISA for the qualitative detection of *Dirofilaria immitis* antigen, anti-*Ehrlichia canis* antibody, anti-*Borrelia burgdorferi* antibody and anti-*Anaplasma phagocytophilum/Anaplasma platys* antibody in canine serum, plasma or whole blood (Anigen Rapid CaniV-4 Test Kits, Bionote). The sensitivity (Anaplasma: 88.5%, Lyme: 93%) and specificity (Anaplasma: 97.1%, Lyme: 98%) of the rapid ELISA test kit were found to be sufficient for diagnostic accuracy. However, there is limitation of detecting *Borrelia* antibodies using a commercial rapid in-clinic ELISA test, *Borrelia* antibodies were evaluated by an immunofluorescent antibody testing (IFAT- MegaScreen Fluoborrelia, MegaCor Diagnostik GmbH, Austria) and cutoff titer was accepted as a 1:64 (sensitivity, 90%; specificity, 98.6%). In the control group, dogs were considered healthy based on clinical, haematological and serum biochemistry profiles as well as negative test results of the rapid test.

Blood samples were collected from the cephalic vein before treatment and then placed into special tubes with and without anticoagulant (BD Vacutainer CAT and K3E 3.6 Mg, Plymouth, UK) to perform haematological (complete blood cell count with VetS-

can<sup>®</sup> HM5, Abaxis) and serum biochemistry analyses (serum C-reactive protein [CRP], haptoglobin [Hp] and ferritin levels). The samples were centrifuged, and the serum samples were separated into cryo tubes (CryoClear<sup>™</sup> Cryogenic Vials, Globe Scientific, USA). Then, frozen samples were stored (-80 °C) until proteomic analysis, which was performed in less than three months after the final patient sampling.

All the procedures made were approved by the Local Ethical Committee of Bursa Uludag University (24.09.2013/2013-14/07).

### CRP, Hp and Ferritin Measurements

Frozen-packed serum samples were sent to the Laboratory of Clinical Pathology, Faculty of Veterinary Medicine, University of Murcia, Spain, for C-reactive protein (CRP), haptoglobin (Hp), and ferritin measurements with the same techniques as reported previously (Karnezi et al, 2016; Martinez-Subiela et al, 2005).

### Proteomic Analysis

These procedures of two-dimensional protein electrophoresis (2D-PAGE), high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and protein identification were performed as reported in detail in our previous article (Escribano et al, 2016). These procedures are briefly explained below.

### 2D-PAGE and image analysis

Pooled serum samples from each group were prepared. The Bradford assay (Sigma-Aldrich, USA) was used to measure protein concentrations in serum samples to standardize the total protein concentrations of each animal in each pool. Isoelectric focusing was performed in a Protean IEF Cell (Bio-Rad). Two-dimensional protein electrophoresis (SDS-PAGE) was run using 12% polyacrylamide gels prepared for the Protean II XL Multi Cell (Bio-Rad). Protein separation was performed with the following 2-step program. Scanning of the stained gels was performed in an ImageScanner II (GE Healthcare Europe GmbH) and evaluated using specific 2D software (Image Master 2D Platinum 7.0, GE Healthcare Europe GmbH). Images of all patient samples were digitalized and aligned to identify differentially expressed protein spots (ANOVA  $P < 0.05$ ) between groups.

### HPLC-MS/MS analysis

Spots were cut out and in-gel digested with trypt-

sin, as described in a previous study (Pantchev, 2010). The separation and analysis of the tryptic digests of the samples were performed with an HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, USA) equipped with a  $\mu$ -well plate auto sampler and a capillary pump and connected to an Agilent Ion Trap XCT Plus Mass Spectrometer (Agilent Technologies, USA) using an electrospray (ESI) interface.

### Protein identification

The data analysis program for Liquid Chromatography/Mass Selective Detector (LC/MSD) Trap Version 3.3 (Bruker Daltonik, GmbH, Germany) and Spectrum Mill MS Proteomics Workbench (Rev A.03.02.060B, Agilent Technologies, USA) were used to perform MS data processing. Raw data were extracted under default conditions as follows: unmodified or carbamidomethylated cysteines; sequence tag length > 1; [MH]<sup>+</sup> 50-7000 m/z; maximum charge +7; minimum signal-to-noise (S/N) 25; and finding 12C signals. The MS/MS search against the appropriate and updated NCBI Inr database from *Canis* sp. was performed with the following criteria: identity search mode; tryptic digestion with 3 maximum missed cleavages; carbamidomethylated cysteines; peptide charges +1, +2, and +3; monoisotopic masses; peptide precursor mass tolerance 2.5 Da; product ion mass tolerance 0.7 amu; ESI ion trap instrument; minimum matched peak intensity 50%; and STY phosphorylation, oxidized methionine, and N-terminal glutamine conversion to pyroglutamic acid as variable modifications.

### Statistics

Descriptive statistical procedures and software (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA) were used to perform statistical analysis. The CV of the assay was calculated as SD divided by the mean value of analysed replicates (100%). Linear regression analyses were performed to assess linearity under dilution. Because of the small sample size, the changes in analytes among healthy dogs and dogs with anaplasmosis, Lyme disease and dual infection were evaluated by the nonparametric Kruskal-Wallis test.  $P < 0.05$  was considered significant.

## RESULTS

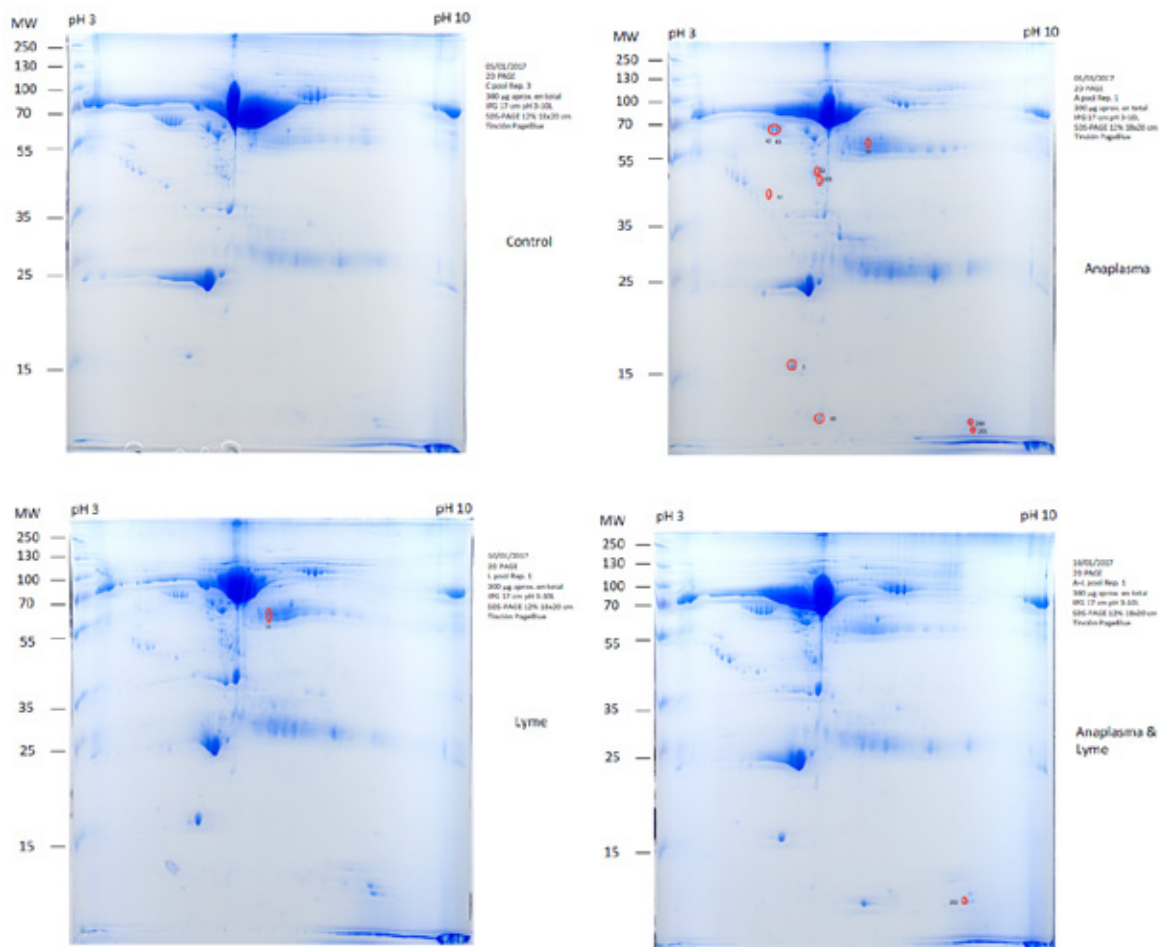
Selected haematological and acute phase proteins were evaluated for all groups (Tables 1 and 2, respectively). CBC results except platelet count in infected

dogs did not differ statistically compared to those of controls (Table 1). The platelet count was significantly lower ( $P < 0.01$ ) in infected dogs (Group-1, Group-2 and Group-3) than in healthy controls. The decrease in platelet count was more severe in dogs with anaplasmosis ( $120 \pm 22 \times 10^3/\mu\text{L}$ ) than in dogs with Lyme disease ( $185 \pm 33 \times 10^3/\mu\text{L}$ ) and dual infected dogs ( $140 \pm 28 \times 10^3/\mu\text{L}$ ) (Table 1).

Serum levels of CRP, Hp and ferritin in infected dogs were significantly higher ( $P < 0.05 - 0.001$ ) than those in control dogs (Table 2). There were no statistically significant differences in these parameters among the three groups of infected dogs.

2DE Gel spots of differentially expressed proteomes were evaluated in dogs with Anaplasmosis, Lyme Disease and dual infection of both, and healthy

dogs. 2DE image analysis showed 57 differentially expressed protein spots between infected animals and controls. Of those, 10 spots were evaluated for identification by MS (Figure 1). Compared to those in control dogs, VDBP, GP9 and KGN-1 levels decreased, while Hp and Ig heavy chain levels with different spots increased in dogs with both diseases. Apo-A1 levels decreased in dogs with anaplasmosis, Lyme disease and dual infections compared to those in control dogs. Serum clusterin levels decreased in dogs with anaplasmosis but were not differentially expressed in dogs with Lyme disease or dual infections compared to those in control dogs. Calpain-3 content decreased in dogs with anaplasmosis and Lyme disease. In dual infection dogs, levels of VDBP, GP9 and KGN-1 decreased, but alpha-1-acid glycoprotein (AGP), Hp and Ig heavy chain levels increased compared to those in control dogs (Table 3).



Vitamin D binding protein: 105, Hemopexin: 34, Apolipoprotein A1: 15, Ig Heavy chain: 25, Haptoglobin-like: 54, Calpain 3: 104, Glycoprotein 9: 42, Kininogen-1: 43, Clusterin: 13, Alpha-1-acid glycoprotein: 46.

**Figure 1.** 2DE Gel spots of differentially expressed proteomes in dogs with Anaplasmosis, Lyme Disease and dual infection of both, and healthy dogs

**Table 1.** Selected hematological and physiological values of the dogs in each group (mean  $\pm$  SEM) of this study

Parameters	Dogs with Lyme Disease (n=5)	Dogs with Anaplasmosis (n=5)	Dual Infected dogs (n=5)	Healthy Control (n=5)
WBC $\times$ 103/ $\mu$ l	11.3 $\pm$ 1.4	9.8 $\pm$ 1.7	10.2 $\pm$ 1.8	17.2 $\pm$ 3.4
RBC $\times$ 109/ $\mu$ l	6.5 $\pm$ 0.4	6.3 $\pm$ 0.4	5.9 $\pm$ 0.8	7.3 $\pm$ 0.5
HCT %	44.2 $\pm$ 0.2	38.2 $\pm$ 2.5	42.3 $\pm$ 2.1	43.9 $\pm$ 1.7
PLT $\times$ 103/ $\mu$ l	185 $\pm$ 33*	120 $\pm$ 22**	140 $\pm$ 28**	315 $\pm$ 17
BW (kg)	17.0 $\pm$ 2.5	24.4 $\pm$ 2.2	19.2 $\pm$ 2.8	17.2 $\pm$ 3.4

Compared to Control Group \* P<0.01 and \*\* P<0.001

WBC: White blood cell, RBC: Red blood cell, HCT: Hematocrit, PLT: Platelet, BW: Body weight

**Table 2.** Selected acute phase protein concentrations of the dogs in each group (mean  $\pm$  SEM) of this study

Parameters	Dogs with Lyme Disease (n=5)	Dogs with Anaplasmosis (n=5)	Dual Infected dogs (n=5)	Healthy Control (n=5)
CRP $\mu$ l/ml	30.6 $\pm$ 15.2***	25.9 $\pm$ 4.8*	25.5 $\pm$ 4.3*	2.0 $\pm$ 0.2
Hp mg/ dL	3.8 $\pm$ 2.4*	3.5 $\pm$ 1.8*	3.9 $\pm$ 1.8 *	1.5 $\pm$ 0.5
Ferritin $\mu$ g/L	379 $\pm$ 165**	453 $\pm$ 165**	153 $\pm$ 54	113 $\pm$ 40

Compared to Control Group \* P<0.05 \*\* P<0.01 \*\*\* P<0.001

CRP: C reactive protein, Hp: Haptoglobin

**Table 3.** Identification and characterization of the spots differentially expressed in dogs with Anaplasmosis, Lyme Disease and dual infection of both, and healthy dogs

Match ID	Region ID	Dogs with anaplasmosis	Dogs with Lyme Disease	Dual infected dogs	Biological Function
105	VDBP	Decrease***	Decrease***	Decrease***	Vitamin D transport and storage
34	Hemopexin	Increase***	Increase***	ND	Inflammation and hemoglobin metabolic process
15	Apo-A1	Decrease**	Decrease**	Decrease**	Immunity, inflammation, apoptosis, lipid metabolism
25	Ig heavy chain	Increase*	Increase*	Increase*	Inflammation
54	Haptoglobin-like	Increase*	Increase*	Increase*	Inflammation, antioxidant activity and hemoglobin binding molecular function
104	CAPN3	Decrease***	Decrease***	ND	Cellular response to sodium and calcium ions
42	GP9	Decrease*	Decrease*	Decrease*	Coagulation
43	KNG-1	Decrease*	Decrease*	Decrease*	Negative regulation of blood coagulation
13	Clusterin	Decrease*	ND	ND	Inflammation and protein stabilisation
46	Alpha-1-acid glycoprotein	ND	Increase*	Increase*	Regulation of acute phase inflammatory response and negative regulation of interleukin-6 production

Compared to Control Group \* P<0.05 \*\* P<0.01 \*\*\* P<0.001

VDBP; Vitamin D binding protein, Apo-A1; Apolipoprotein A1, CAPN3; calpain 3, GP9; Glycoprotein 9, KNG-1; Kininogen-1, ND; not determined.

## DISCUSSION

This study showed that many serum proteins might be expressed, upregulated or downregulated, in dogs naturally infected with *A. phagocytophilum* (anaplasmosis) and *B. burgdorferi* (Lyme disease). These proteins could be used to understand the detailed pathophysiology of the diseases and provide advantages as possible biomarkers revealing the diagnostic and therapeutic steps of the cases.

In this study, thrombocytopenia and an activated serum acute phase reaction (elevated CRP, Hp, and ferritin levels) were observed in dogs with anaplasmosis and Lyme disease. Compared to those in control dogs, the increase in the serum CRP level was more severe (max 15-fold) than the increase in serum Hp and ferritin levels (maximum 2-3-fold) in infected dogs. In previous studies, serum CRP levels increased in dogs with *Anaplasma* (Pantchev, 2010; Dyachenko

et al, 2012; Baric Rafaj et al., 2013) and Babesia-infected dogs (Kuleš et al, 2016). We also observed that dual infection did not aggravate thrombocytopenia and inflammation in this study. These results showed that the severity of thrombocytopenia and the increase in serum acute phase proteins, especially CRP, could not be used to discriminate between VBD and dual infections.

2DE of pooled samples showed 57 differentially expressed protein spots between infected animals and controls. Of those, 10 proteins were evaluated by MS, while others, such as Hp, VDBP and Apo A-1, were already identified in previous reports (Escribano et al, 2016; Kocatürk et al, 2016; Kuleš et al, 2014; Miller et al, 2014).

Hp is one of the positive acute phase proteins in dogs with a status of inflammation that also has antioxidant activity and haemoglobin-binding molecular function. Increased oxidative stress in dogs with both VBDs may have a positive role in increased haemoglobin uptake in the bloodstream, triggering Hp upregulation (Vickers et al., 2010). In this study, the observed increases in serum Hp levels showed the activation of the inflammatory response of the host organism against vector-borne pathogens, compatible with a previous study carried out in dogs with ehrlichiosis (Escribano et al., 2017).

In this study, the serum level of VDBP decreased in dogs with anaplasmosis, Lyme disease or both. VDBP is expressed in the liver and plays important role(s) in vitamin D transport and storage. Decreased VDBP content in dogs with VBDs may be related to decreased synthesis with/or excessive use of proteins in the liver that can have an impact on circulating vitamin D levels, leading to negative effects on the immune response of the host against infections, such as VBDs (Uysal et al., 2015). Possible tissue and/or organ injury as well as coagulation activation during both infections in dogs can cause alterations in serum VDBP levels (Bottari et al., 2016; Li et al., 2016). The overuse of vitamin D to neutralize the overproduction of ferritin and transferrin in response to VBDs may also be the reason for the decrease in VDBP in this study (Escribano et al., 2017, Kules et al, 2014; Kuleš et al., 2016).

Serum Apo-A1 has multiple roles in immunity, inflammation, and apoptosis and plays a vital role in cholesterol-binding and transport activities and cellular cholesterol homeostasis (Kuleš et al., 2016, Man-

garaj et al., 2015). In the present study, decreased levels of serum Apo-A1 in dogs with Lyme disease were considered a host response to the pathogen. Apo-A1 has been documented as a potential biomarker in treatment monitoring in canine leishmaniasis (Escribano et al., 2016). Excessive use of Apo-A1 via the immune response and organ damage may play a role in the decreased level of serum Apo-A1 in response to anaplasmosis, Lyme disease and dual infections in dogs.

AGP is one of the regulators of the acute phase inflammatory response and negatively regulates interleukin-6 production. In previous studies, an increase in serum AGP was reported to be a useful marker to assess inflammatory status (Escribano et al., 2016, Hagman, 2011) and to differentiate from acute to chronic stages of inflammatory disease (Yuki et al., 2010). In parallel with the literature, in this study, an increased level of serum AGP was detected in both the single and dual infection groups compared to that in the healthy control group (Escribano et al., 2016, Hagman, 2011, Yuki et al., 2010).

KNG-1 binds to kallikrein to release the proinflammatory mediator bradykinin, a proinflammatory peptide, and has antithrombin function and antiadhesive properties (Yousef et al., 2003; Bryant et al., 2009). However, kallikrein is a chemotactic factor for neutrophils (Kaplan et al., 1972) and monocytes (Gallin et al., 1974) shown to cause neutrophil aggregation (Schapira et al., 1982). In our study, decreased levels of GP9 were seen in dogs with dual and single infections. GP9 functions as a von Willebrand factor (vWF) receptor and mediates vWF-dependent platelet adhesion to blood vessels (Ware et al., 2002; Ruggeri et al., 2003; Garcia-Martinez et al., 2012). To the authors, downregulation of both KNG-1 and GP9 might signal an impaired inflammatory response and coagulation cascade due to vector-borne diseases evaluated in our study.

Clusterin (apolipoprotein J) is one of the chaperone proteins secreted under stress conditions, such as cell death, tumour progression and neurodegenerative disorders. It has been reported that increases in serum and urine clusterin content are early diagnostic tools to evaluate renal function (Garcia-Martinez et al., 2012, Zhou et al., 2014). In our study, decreased serum clusterin levels were detected in dogs with anaplasmosis compared to those in control dogs. As mentioned in a previous study (Miller et al., 2014), the possible reason for the decreased level of serum

clusterin in Group-1 might be the excessive use of the protein due to the inflammatory oxidative stress status in VBDs.

CAPN3, which is found within muscle cells in structures called sarcomeres, belongs to the calpain superfamily and is predominantly expressed in skeletal muscle. In human medicine, a previous report showed that CAPN3 has an active role in type 2 diabetes mellitus (Harris et al., 2006). CAPN3 was also shown to be highly expressed by melanoma cells, causing cell proliferation and stimulating cell death (Weeraratna et al., 2004). However, downregulation of serum CAPN3 has been reported in dogs with mitral valve disease (Locatelli et al., 2017). In our study, downregulation of serum CAPN3 was seen in dogs with only anaplasmosis or Lyme disease. Unlike in human studies, CAPN3 seems to behave differently in dogs with inflammation; therefore, additional studies are needed to elucidate the behaviour of this proteome constituent.

Serum proteomic changes pre and post treatment were not evaluated in this study. Investigators may further evaluate this hypothesis to compare the diseased and treated expressional serum proteome status of dogs with anaplasmosis and/or Lyme disease. Serum AGP content may be a reliable biomarker of inflammatory disease in dogs and may be useful to differentiate acute and chronic phases of disease.

A major limitation of this study is the small number of patients in the groups, limiting the potential to identify proteins due to biological inter-individual variation. Nevertheless, the proteome is commonly studied in a minimum of three biologically similar samples in order to adequately determine the biological variations in the proteome in veterinary studies (Westermeier et al., 2008). Even in larger numbers of patients, the probability of false positive and false negative results due to biological variation could occur when samples are pooled. For that reason, only proteins identified in all five samples were chosen for statistical evaluation and bioinformatic analysis to lower the inter-individual variation in this study (Sadiq et al., 2008; Kristensen et al., 2014). The main advantage of pooling samples is to find the main biomarkers differentiating each group of patients. Pooling samples from healthy and diseased groups reduces the sample size while maintaining a high degree of confidence in the data; however, it also eliminates the estimation of inter-individual variation within each group and can mask outliers that can reduce the applicability of

the biomarker upon validation (Orton and Douchette, 2013). Therefore, this study may be considered a pilot (initial) study using pools, and in a future study, these findings should be validated by individual sample analysis. Another limitation of our study is the use of a commercial rapid in-clinic ELISA (Anigen Rapid CaniV-4 Test Kits, Bionote) despite having sufficient sensitivity (Anaplasma: 88.5%, Lyme: 93%) and specificity (Anaplasma: 97.1%, Lyme: 98%) for the diagnosis of those two diseases. Besides, *Borrelia* antibodies were evaluated by an IFAT in this study (sensitivity, 90%; specificity, 98.6%). However, panellists of the ACVIM-updated consensus statement of Lyme borreliosis in dogs and cats did not recommend whole-cell ELISA, IFAT or Western blot testing as a result of possible cross-reactions with other spirochetal infections or IgM versus IgG antibody testing due to not showing acute illness before seroconversion in dogs (Littman et al, 2018). Therefore, in this study, a multiplex assay detecting OspC antibodies might be accepted as a more sensitive and reliable test indicating recent exposure or re-exposure to Lyme borreliosis in dogs. This study focused on changes in serum proteomes differentiating between healthy and diseased dogs; however, serum protein analysis could have been performed before and after treatment to monitor treatment efficiency.

Proteomics studies of VBD are important for supplying additional information for a better understanding of pathology and for the evaluation of the outcome of these diseases. This study showed that the levels of many proteins might be changed in anaplasmosis and Lyme disease, and determining the role of these proteins in many biological processes (acute phase response, immunological reactions, transport, oxidative stress, apoptosis, and calcium, iron and lipid metabolism) can supply benefits during diagnostic and therapeutic procedures for both.

## CONFLICT OF INTEREST

None of the authors has any financial or personal relationships that could inappropriately influence the content of the paper.

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