Original Article

Diagnostic value of C-reactive protein and soluble urokinase plasminogen activator receptor in canine monocytic ehrlichiosis

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Abstract

Canine monocytic ehrlichiosis is a vector-borne disease that can induce renal failure in dogs. In this study we aimed to describe the potential diagnostic efficiency of CRP and suPAR in dogs infected with *E. canis* and the association between suPAR and CRP with renal failure. For this purpose, the study consisted of a total of 17 dogs, 8 of infected with *E. canis* and 9 of healty. For evaluating renal failure, plasma urea-creatinine and UPC were determined and also measurements of plasma CRP and suPAR were analysed with ELISA test kits. Mean CRP, suPAR, urea, creatinine and UPC levels of the infected dogs were significantly higher compared with healthy ones ($P<0.01$) but the correlation of all parameters with each group was not significant ($P>0.05$). As to be a result of ROC curve analysis and Youden's index, the cut-off values for diagnosis of ehrlichiosis were identified to be 9.68 mg/L for CRP and 168.2 ng/L for suPAR. Furthermore, the specificity and sensitivity of both markers according as a potential biomarkers cut-off values were determined higher. In conclusion, traditionally CRP and currently suPAR can be considered as potential biomarkers and have a diagnostic significance following inflammation in canine ehrlichiosis.

Keywords: CRP, suPAR, kidney failure, inflammation, *E. canis*

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Introduction

Canine monocytic ehrlichiosis (CME) vector-borne disease is caused primarily by *Ehrlichia canis* (Neer *et al*., 2002, Sainz *et al*., 2015). Distribution of CME has been reported all over the world especially in tropical and subtropical regions such as Turkey (Near *et al*., 2002; Aysul *et al*., 2012; Çetinkaya *et al*., 2016; Gültekin *et al*., 2017).

Ehrlichia canis demonstrates systemic spread with infected monocytes thereby it may effect multiple organs including the kidneys (Silva *et al*., 2016; Anusaksathien *et al*., 2019; Ziliani *et al*., 2019) with a severity that is affected in varying degrees at different stages of the infection (Ziliani *et al*., 2019). Considering renal damage according to the stage of infection, in the acute stage glomerulopathy consists of proteinuria (Codner *et al*., 1992; de Castro *et al*., 2004); in the subclinical stage, renal damage is caused by immune complex accumulation concomitant with glomerulosclerosis, basement membrane thickness and mesenchymal proliferation (Crivellenti *et al*., 2015); and finally, in the chronic stage, glomerulonephritis might occur through immune complex accumulation (Iqbal and Rikihisa, 1994) and also prolonged exposure to *E. canis* may stimulate insidious glomerulonephrotic alteration leading to severe renal disease in infected dogs (Heiene *et al*., 2007; Goldstein *et al*., 2013). *Ehrlichia spp*. infection has a potential for glomerular disease as it causes glomerular injury with an accumulation of immune complex. Additionally, acute kidney injury related to CME is more common than chronic renal failure (Suwanpakdee, 2018) so that risk of complications and mortality increases (Suwanpakdee, 2018).

It is seen that inflammation is an important factor for the initiation and progression of renal failure in dogs in that some essential cytokines expression occur and change renal function (Nentwig *et al*., 2016). Furthermore, this stimulation causes infiltration of inflammatory cells to the kidneys as a results to occurring additionally cytokines response (Akcay *et al*., 2009; Nentwig *et al*., 2016). In this regard the acute phase protein, C-reactive protein (CRP), is conventionally a biomarker of inflammation that exists at higher levels in serum in consequence of the response to proinflammatory cytokines releasing from inflammatory cells related to infection (Yamashita *et al*., 1994; Niehues, 2018). It is stated that there has been an important relationship between CRP and renal markers (serum creatinine, urine protein/creatinine, proteinuria) (Raila *et al*., 2011) and an increase of CRP related to inflammatory damage in dogs experimentally infected with *E. canis* (Rikihisa *et al*., 1994; Shimada *et al*., 2002).

Another current inflammatory biomarker (Degryse, 2003; Mondino and Blasi, 2004) is cell surface receptor urokinase-type plasminogen activator receptor (uPAR) whose structure forms a glycosylphosphatidylinositol-anchored protein and it is specific to urokinase plasminogen activator (uPA) as part of the cell surface protease system (Montuori *et al*., 2013). uPA and uPAR are mainly released from blood cells such as neutrophils, monocytes, macrophages and activated T cells, and are involved in various immune functions such as cell adhesion, migration, differentiation and proliferation. In inflammation and infectious responses, the amount of uPAR increases in leukocytes and cell migration is activated for proteolysis of the extracellular matrix by binding uPA to uPAR (Collen, 1999; Rosso *et al*., 2011; Genua *et al*., 2014). uPAR interacts with integrins in the extracellular matrix, increasing cell adhesion and migration. At the same time, binding to integrins induces cell differentiation and proliferation, causing an increase in intracellular signaling (Ragno, 2006). As a result of inflammatory stimulation, proteases such as chemotrypsin, phospholipase C and uPA cause the release of uPAR from the cell surface to the circulation with the formation of a soluble form of urokinase plasminogen activator receptor (suPAR) (Collen, 1999; Rosso *et al*., 2011; Genua *et al*., 2014). suPAR can be found in several body fluids and has a similar function to uPAR (Thunø *et al*., 2009; Rosso *et al*., 2011). suPAR is a promising new biomarker for evaluating inflammation, organ injury and prognosis in several disorders (Zimmermann *et al*., 2012; Hamie *et al*., 2018; Higham *et al*., 2020; Huang *et al*., 2020) including kidney failure in humans (Hayek *et al*., 2015;2020 Hall a *et al*., 2018; Hamie *et al*., 2018). Thus, it is released from immune cells during inflammation and migrates to kidney glomerulus. In there, it activates αVβ3 integrin on podocytes as a result increasing suPAR leading to a change of podocyte function causing proteinuria (Wei *et al*., 2011; Kriz and Lembley, 2015).

Traditionally C-reactive protein and currently suPAR are independently inflammatory biomarkers for predicting inflammatory conditions (Degryse, 2003; Mondino and Blasi, 2004; Lyngbæk *et al*., 2013; Basbug *et al*., 2020). Therefore, following CRP and suPAR might be benefit during disease progress and treatment.We hypothesized that both biomarkers represent a different approach to inflammation associated with renal failure in CME. In this study we aimed to describe the potential association between suPAR and CRP, to compare these as diagnostic markers of infection CME.

Materials and Methods

Sampling and animal material: A total of 17 dogs, 8 infected with *E. canis* and 9 control, were included in the study. Data of dogs was recorded in different breeds and sexes, age ranging between 2.5 and 8. The infected group was determined to be positive for *E. canis* during its routine evaluation at the Faculty.

The infected dogs were selected from among the routine patients which were admitted to Faculty hospital for diagnosis and treatment with various complaints and co-infected dog with other vector borne diseases (Dirofilariosis, Anaplasmosis, Lyme Disease, Leishmaniasis) were excluded and only *E. canis* infected dog were enrolled. Similarly, the control group was selected from among healthy dogs of those routine controls related to vaccination and/or check-up. All of the control group were checked for *E. canis* during routine control and determined negative for *E. canis*. The diagnosis of ehrlichiosis was performed by rapid test kits (IDEXX SNAP® 4Dx® Plus Test, MVM Medikal, Ankara) and confirmed with indirect

fluorescence antibodies test (IFAT) in which titers over 1:240 was evaluated positive for CME.

Taking blood sample: Blood samples were obtained from Vena cephalica into anticoagulant and serum tubes. Urine samples were collected by spontaneous urination into sterile tube with a minimum of 10 ml.

Classification of novel 'acute or chronic kidney disease': All cases were enrolled in the present research as if acute onset of selected findings in harmony with acute kidney injury (i.e., vomiting, lethargy, anorexia) along with azotemia as detected by serum creatinine levels >1.6 mg/dL), as reported previously (Dunaevich *et al*., 2020). Relevant data was not shown because those parameters were supporting criteria, not the purpose of the study. In addition even if one of the above mentioned criteria was evident, for establishing a tentative diagnosis of chronic kidney disease: (1) prior proof of chronic kidney disease determined by persistent elevation of serum creatinine increase, accompanied by 25% or above contemporaneous elevation in contrast to baseline values; (2) abdominal ultrasonographic interpretation as in line with chronic kidney disease as evidenced by two or more criteria: elevated renal echogenicity, significantly declined renal corticomedullary differentiation, altered renal size/asymmetry, and cystic structure/irregular kidney contour (Dunaevich *et al*., 2020) was required.

Determination of plasma urea-creatinine and urine protein/creatinine ratio: Heparin samples were separated to plasma by centrifuging at 1500 rpm for 10 minutes and as part of biochemical analysis, urea and creatinine were measured with an autoanalyzer (RX Daytona +, Randox, Simre Medikal, Ankara) at Faculty. The remaining plasma samples were frozen at − 80 °C until analysis. Likewise, immediately after the collection of urine samples, urine protein/creatinine ratio (UPC) was determined by urine strips (Uritest 11G, China) as determined before (Moore *et al*., 1991; Marynissen *et al*., 2017; Meindl *et al*., 2019).

CRP ve suPAR analysis: C-reactive protein and suPAR analysis was determined in heparinized plasma using sandwich ELISA in accordance with the instructions of the manufacturer (Yehua Bio. Tech., Shangai) with frozen samples that were being melted under suitable condition at analysis time.

Statistical analysis: All data was expressed as mean and standart error. Normality test of data was evaluated with Kolmogorov-Smirnov test. Comparisons between groups were conducted by independent samples T test due to continuous variables found within a normal distribution.

For determining the diagnostic potential of CRP and suPAR in dogs with ehrlichiosis, receiver operating characteristic (ROC) curves were used. For this purpose, the area under curve (AUC) and cut-off values were assessed according to classifying of AUC values as 0.91–1.00: excellent, 0.81–0.90: good, 0.71–0.80: fair, 0.61–0.70: poor and 0.51–0.60: fail. The sensitivity (Se) and specificity (Sp) with positive and negative predictive values of CRP and suPAR were evaluated at their 95% confidence intervals and at a significance level of $P < 0.05$ for the cut-off level determining with ROC as well as the Youden's index. For all analyses SPSS (22.0, IBM) and MedCalc (MedCalc Software, Belgium) were used.

Ethical approval: Protocol of study did not have to be approved by an animal welfare ethical committee because collecting of samples was realized within routine evaluation of dogs at the hospital. For using routine samples and results of dogs in the study, owner permission was received.

Results

Clinical evaluation: On admission to hospital, dogs in the group infected with CME were observed with various but common clinical findings of those waxing and waning with all infected dogs presenting with generalized lymphadenopathy, fever (39.7 ^{0}C) and corneal opacity in 1/8, weight loss in 2/8, hind limb weakness and/or epistaxis in 4/8, hematuria and oedema of in 1/8 . There was no pathological condition as result of routine examination related to hemogram, biochemical and clinical examination in the healthy group. All dogs were classified as acute or chronic kidney disease based on laboratory findings as detected by Dunaevich *et al*. (2020).

Data evaluation: Mean values of the CRP, suPAR, UPC, urea and creatinine levels of healthy and infected dogs are presented in Table 1 with inter-group significance. Comparison of all parameters between groups shows group statistically significant differences. Distribution of CRP and suPAR between the healthy and infected group are submitted in Fig. 1. It was found that the CRP and suPAR of infected dogs with *E. canis* were significantly higher compared with healthy ones (Table 1, Fig. 1).

For assessing the prognostic potential of CRP and suPAR in ehrlichiosis, ROC analysis was used. Given evaluation in prognostic indicators of the CRP and suPAR, all of cut-off, sensitivity, specificity and AUC values were shown in Table 2 and only for suPAR in Fig. 2-3. The cut-off values were revealed as 9.68 mg/L for CRP and 168.2 for suPAR.

Correlation of CRP and suPAR was negative but not significant in the infected group r=0.67, P=0.110 (P>0.05) and positive but not significant in the healthy dogs r=0.488, P=0.183 (P>0.05). Similarly, there was no correlation between the renal marker (urea, creatinine and UPC) and CRP or suPAR in the infected group (P>0.05).

Table 1 The CRP, suPAR, UPC, urea and creatinine levels of healthy and infected dogs (Mean±Standart error mean)

	Infected group (n=8)	**Healthy group (n=9)**	***P-value***
CRP (mg/L)	122.53 ± 8.62	4.3 ± 1.08	<0.01
suPAR (ng/L)	355.32 ± 71.2	111.92 ± 17.43	<0.01
UPC	3.83 ± 1.18	0.1 ± 0	<0.01
Urea (mg/dL)	1.63 ± 0.36	0.53 ± 0.41	0.004
Creatinine (mg/dL)	82.25 ± 11.42	29.11 ± 3.67	0.001

CRP; C-reactive protein, uPAR; urokinase-type plasminogen activator receptor, UPC; urine protein/creatinine.

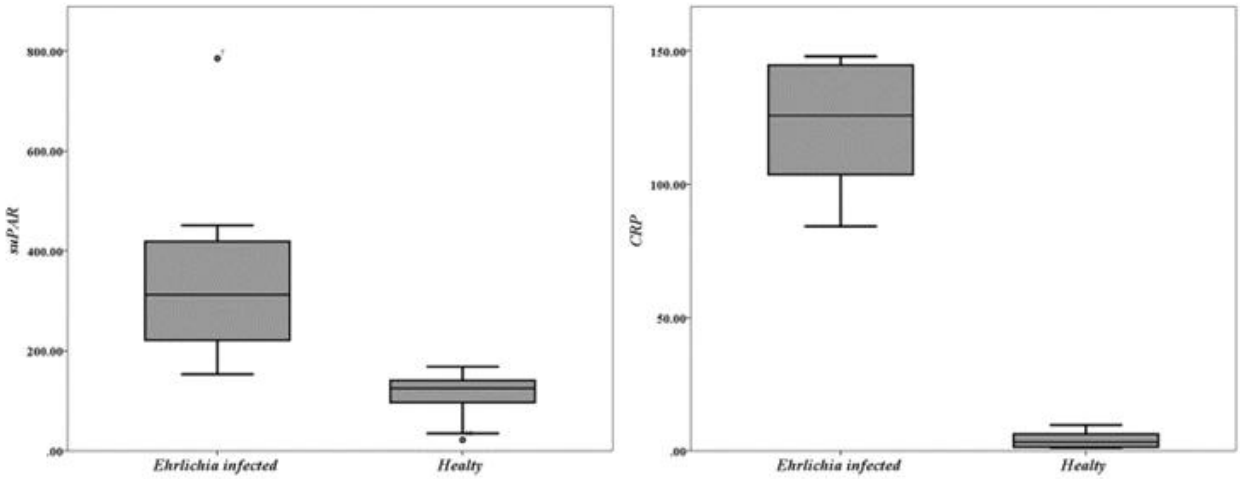

Figure 1 Box plots of plasma CRP and suPAR concentrations in healthy and *E. canis* infected dogs. The boxes show 25 and 75 percent slices, whiskers show 10 and 90 percent slices. The line in each box indicates the median.

Table 2 The cut-off levels, specificity, sensitivity, and AUC values of CRP and suPAR for diagnosis canine ehrlichiosis

Parameter	**AUC**	**95% confidence interval**	**Cut-off**	**Specificity**	**Sensitivity**	**Youden's index**	***P value***	**SEM**
CRP (mg/L)	1.000	0.805 – 1.000	>9.68	100.00	100.00	1.0000	<0.0001	0.000
suPAR (ng/L)	0.972	0.759 – 1.000	>168.2	100.00	87.50	0.8750	<0.0001	0.0333

CRP; C-reactive protein, uPAR; urokinase-type plasminogen activator receptor, AUC; area under the curve, SEM; standard error of mean

The cut-off values of CRP and suPAR were determined by using ROC curve analysis and Youden's index

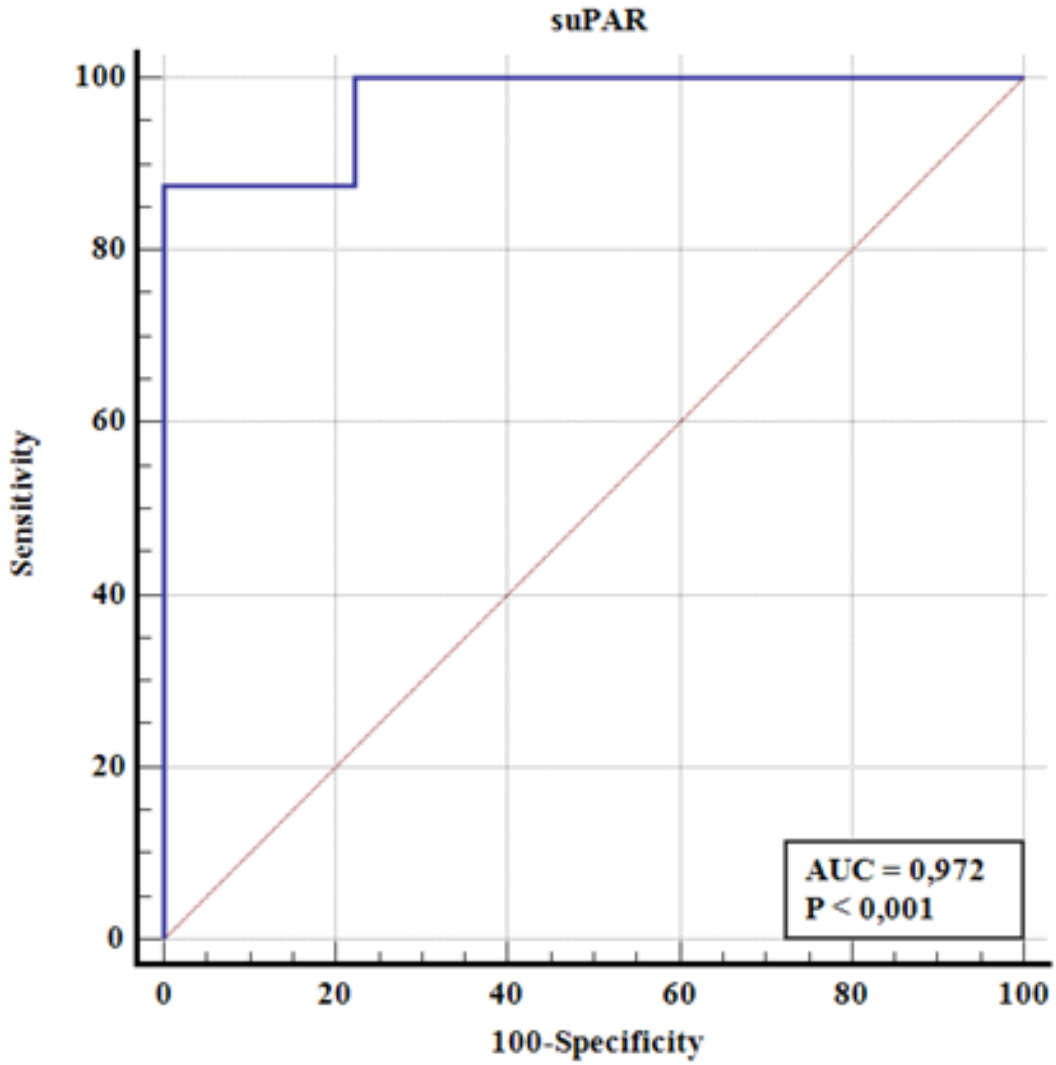

Figure 2 ROC curves for suPAR in healthy and *E. canis* infected dogs. AUC and 95% CI for ehrlichiosis is 0.972 (95% CI: 0,759 - 1,000) for suPAR.

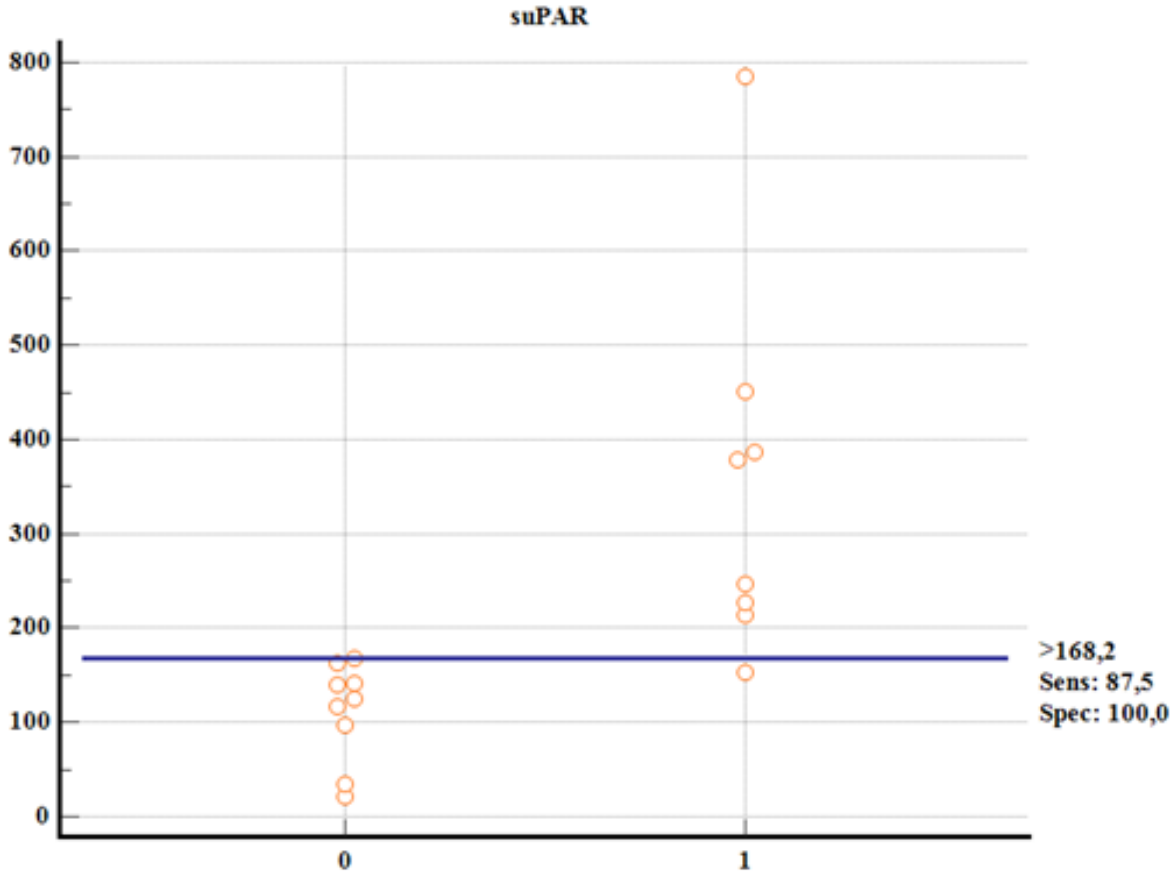

Figure 3 Dot plot diagram showing cut-off value, specificity and sensitivity of suPAR obtained from dogs infected with *E. canis*

Discussion

Canine monocytic ehrlichiosis may lead to acute or chronic renal failure resulting from glomerular injury with accumulation of immune complex (Goldstein *et al*., 2013; Crivellenti *et al*., 2015; Silva *et al*., 2016; Anusaksathien *et al*., 2019; Ziliani *et al*., 2019). It is thought that inflammation is a part of the initiation and progression of renal failure (Nentwig *et al*., 2016). Consistent with this, we aimed to investigate CRP and suPAR as an independently inflammatory biomarker (Degryse, 2003; Mondino and Blasi, 2004; Lyngbæk *et al*., 2013; Basbug *et al*., 2020) in dogs infected with CME and relation with kidney failure.

Considering the relation of CME with inflammation (Yamashita *et al*., 1994; Niehues, 2018), CRP is found to be higher than normal in dog infected with *E. canis* (Rikihisa *et al*., 1994; Shimada *et al*., 2002; Mylonakis *et al*., 2011; Karnezi *et al*., 2016). As consistent with other research, in our study indicated that plasma CRP levels were higher in the infected group with mean 123.53 mg/L (P<0.01). Additionally, the cut-off value of CRP was determined as 9.68 mg/L for infected dogs with Sp 100% and Se 100%.

As also in relation to our research, it is stated that increasing suPAR can be used as a predictor marker for acute and chronic kidney failure (Hayek *et al*., 2015;2020 Hall *et al*., 2018; Hamie *et al*., 2018) but relevant data is lacking in dogs. Similary, only one research has been encountered regarding its alteration in vector-borne diseases (Kuleš *et al*., 2014) unlike CRP (Venco *et al*., 2014; Karnezi *et al*.,2016; Pardo-Marin *et al*., 2020). In this context, alteration regarding suPAR levels and the relationship between suPAR and renal failure due to ehrlichiosis was firstly elucidated with this study. It was found in our study that mean suPAR concentration of infected dogs were significantly higher than controls regarding CRP (P<0.01). Besides, the cutoff value of suPAR was determined with 168.2 ng/L for healthy and infected dogs with Sp 100% and Se 87.50%. In our study indicated that suPAR and CRP might be used as a marker for the detection of inflammation in dogs infected with *E. canis*. however, both markers were not appeared to be associated with renal markers to be significantly higher in dogs infected with *E. canis* than healthy ones (P<0.01).

As to veterinary medicine, CRP and suPAR have been scrutinized together in limited number of studies

(Rafaj *et al*., 2016; Kučer *et al*., 2018; Basbug *et al*., 2020). Despite what is mentioned above, there is no study investigating suPAR in ehrlichiosis in both medicines. suPAR in comparation to CRP has more advantage with measuring even in small levels in plasma (Rønne *et al*.,1995; Stephens *et al*., 1997), not affected by fasting and diurnation (Sier *et al*., 1999) and being resistant to thawing and freezing procedure (Riisbro *et al*., 2001). In one study related to biomarkers of inflammation in obese dogs was shown that IL-6 and hsCRP as an inflammatory marker were higher in obese dogs with $P = 0.001$; $P = 0.027$ but suPAR was decreased as gain weight in dogs ($P= 0.002$). In another study was shown that hsCRP and suPAR had a significant and positive correlation for using to diagnosis of canine lymphoma (r=0.75-1.00) (Kučer *et al*., 2018). Basbug *et al*. (2020) indicated that suPAR was found to be higher in dogs with SIRS (1001.79±287.60 ng/L, $P=0.007$) but insufficient to diagnosis with cut-off value as 223.68 ng/L and lower sensitivity (53.3%) by comparation with CRP as 92.90%. In our study, CRP and suPAR did not correlate with each other ($P>0.05$) however, mean CRP and suPAR levels were significantly higher ($P<0.01$) in *E. canis* infected dog and so, ROC curve showed that CRP and suPAR had 100% of Sp with 9.68 and 168.2 cut-off values in infected dogs.

In conclusion, traditionally CRP and currently suPAR can be considered as a potential biomarker and have a diagnostic significance for the following of inflammation in CME. Even so, considering the limitation of our study including the small sample size and not following a long-term prognosis, it is thought that the small sample size influenced the results in determining the efficacy of suPAR in renal failure and further studies performed with a larger population are necessary for determining the relationship between suPAR and renal failure borne to canine ehrlichiosis. And also, the progressing of renal failure changes according to the stages of ehrlichiosis. For this reason, we recommend evaluating suPAR according to the stages as well as to reveal the prognostic effect on treatment in future studies.

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