

Journal of the Hellenic Veterinary Medical Society

Vol 71, No 1 (2020)



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doi: [10.12681/jhvms.22936](https://doi.org/10.12681/jhvms.22936)

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To cite this article:

BASBUG, O., AYDOGDU, U., & AGAOGLU, Z. T. (2020). Neopterin and Soluble Urokinase Type Plasminogen Activator Receptor as Biomarkers in Dogs with Systemic Inflammatory Response Syndrome. *Journal of the Hellenic Veterinary Medical Society*, 71(1), 1945–1952. <https://doi.org/10.12681/jhvms.22936>

Neopterin and Soluble Urokinase Type Plasminogen Activator Receptor as Biomarkers in Dogs with Systemic Inflammatory Response Syndrome

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ABSTRACT. The systemic inflammatory response syndrome (SIRS) is a common cause of mortality in dogs. This study aims to investigate the diagnostic value of markers C-reactive protein (CRP), soluble urokinase-type plasminogen activator receptor (suPAR) and neopterin in dogs with SIRS. The materials of the study consist of 30 dogs with SIRS and 15 healthy dogs that show no clinical symptom, 45 dogs in total. CRP, suPAR and neopterin levels of the serum samples were determined by ELISA using commercial kits. In the study, it was found that CRP, suPAR and neopterin levels of dogs with SIRS were significantly higher ($P < 0.001$, $P < 0.01$ and $P < 0.01$, respectively) than those in the healthy group. The cut-off values for the healthy dogs and dogs with SIRS for CRP, suPAR and neopterin were determined to be 11.20 mg/L, 223.68 ng/L and 3.23 nmol/mL, respectively. At these cut-off values, sensitivity levels were determined as high (92.90%) for CRP, moderate (63.30%) for neopterin, and low (53.30%) for suPAR, and high specificities (93.30%) were determined for all.

In conclusion, it was determined that CRP showed the best performance for the detection of systemic inflammatory response in dogs and neopterin and suPAR could be used as an alternative to CRP. It was also argued that further studies were necessary in order to determine diagnosis and prognosis of diseases.

Keywords: Systemic inflammatory response syndrome, acute phase proteins, dogs

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Date of initial submission: 02-01-2019
Date of revised submission: 10-07-2019
Date of acceptance: 07-11-2019

INTRODUCTION

Systemic inflammatory response syndrome (SIRS) is an immune response against infectious or non-infectious factors (Spapen et al., 2006). Two or more criteria in the findings of body temperature $> 39.4^{\circ}\text{C}$ or $< 37.8^{\circ}\text{C}$, heart rate > 140 beat/minute, respiration rate > 20 /minute, leukocyte $> 16.0 \times 10^9/\text{L}$ or $< 6.0 \times 10^9/\text{L}$ and $> 3\%$ band formation are to be found in order to diagnose SIRS (Torrente et al., 2015). Sepsis is defined as the SIRS developed against infections and is indicated as the most notable cause of death in infectious diseases (Alberti et al., 2003; Martin et al., 2003). Although clinical and diagnostic tests are used in human and veterinary medicine, more sensitive tests are required (Spapen et al., 2006; Nakamura et al., 2008; Mylonakis et al., 2011; Ok et al., 2015; Torrente et al., 2015). Especially in veterinary medicine, the biomarkers that show diagnostic and prognostic values of SIRS or sepsis are not sufficient.

Recent studies state that acute phase proteins can provide diagnostically and prognostically significant information in monitoring SIRS in infectious or non-infectious patients. Acute phase proteins are liver-based glycoproteins formed as a result of acute phase response. Acute phase proteins are called as positive or negative depending on increase (such as C-reactive protein and serum amyloid A) or decrease (such as albumin and transferrin) in their levels during inflammatory states. Acute phase response is a natural defense mechanism that plays a part in limiting the tissue damage and in stimulating recovery after trauma, infection or inflammation (Eckersall and Conner 1988; Tothova et al., 2014). Different researchers reported that C-reactive protein (CRP), which gives non-specific immune responses during the monitoring of infectious diseases, could be used in the diagnosis of infectious diseases. CRP is a liver-based glycoprotein formed as a result of acute phase response. In the studies on veterinary medicine, it was stated that serum CRP, one of type-specific acute phase proteins, may yield significant information in evaluating the clinical presentation of diseases in dogs (Christensen et al., 2014; Ok et al., 2015).

The soluble urokinase-type plasminogen activator receptor (suPAR) is a potential biomarker reported for inflammatory diseases. suPAR is released from neutrophils, endothelial and peripheral mononuclear blood cells and take part in various immune functions such as cell adhesion, migration, differentiation and proliferation. Increased suPAR levels are known as

the determinant of the activation of immune and inflammatory systems, and have prognostic values in various diseases (Kofoed et al., 2008; Eugen-Olsen, 2011). Although it was reported that suPAR might have had diagnostic values in sepsis, lung and malignant diseases (Stephens et al., 1997; Wittenhagen et al., 2004; Eugen-Olsen 2011) no study related to dogs with SIRS in veterinary medicine was found in the literature surveys.

Neopterin [2-amino-4-hydroxy-6-(D-erythro-1', 2', 3'-trihydroxypropyl)-pteridine] is a low molecular weight molecule belonging to the class of pteridines which biosynthetically derives from guanosinetriphosphate (Murr et al., 2002). Neopterin is synthesized by monocytes and macrophages as a result of stimulating interferon-gamma (IFN- γ) released from active T lymphocytes. Neopterin is the sensitive determinant of cell-mediated immunity (Murr et al., 2002; Cesur et al., 2014). Neopterin release commences 3 days before T cell proliferation reaches the maximum level. Neopterin production increases 1 week before specific antibodies emerge. For this reason, neopterin is shown as an indicator of early inflammation (Millner et al., 1998; Berdowska and Zwirska-Korczala 2001). It was determined in the studies on human medicine that neopterin levels rise in viral infections, autoimmune diseases, inflammatory and malignant diseases (Berdowska and Zwirska-Korczala 2001). It is known that neopterin can be used as an indicator in determining the cellular immune response that plays a part in the pathogenesis of various diseases (Murr et al., 2002; Cesur et al., 2014) however, there are very few studies on this matter in the field of veterinary medicine.

It is important to determine reliable test methods that provide information in evaluating diagnosis and prognosis of patients with SIRS. The purpose of this study is to determine CRP, suPAR and neopterin levels of dogs with SIRS.

MATERIALS AND METHODS

Study design and animals

The animal material of the study consisted of 30 (18 females, 12 males) dogs, which were of different species and gender diagnosed with SIRS by clinical symptoms and haematological findings upon having been brought to the Internal Diseases Clinic of the Faculty of Veterinary Medicine of Sivas Cumhuriyet University for examination and treatment, and 15 (8 females, 7 males) healthy dogs, which showed no disease symptom.

The appetite, body temperature, heart rate, respiration rate, respiration type, capillary refill time, dehydration degree, changes in mucous membrane colour and mental status of dogs were examined. By the evaluation of clinical and haematological findings, the dogs diagnosed with SIRS were considered as the patient group of the study.

The study included dogs with at least two of the criteria below for SIRS diagnosis (Torrente et al., 2015).

Body temperature $> 39.4^{\circ}\text{C}$ or $< 37.8^{\circ}\text{C}$

Heart rate > 140 beat/minute

Respiration rate > 20 /minute

Leucocyte $> 16.0 \times 10^9/\text{L}$ or $< 6.0 \times 10^9/\text{L}$ and $> 3\%$ band formation.

10 ml blood samples in total from each dogs were taken from Vena cephalica antebrachii into tubes with anticoagulant (potassium EDTA) and no anticoagulant. Haematological parameters (reference value of leucocyte was taken as $6.0\text{--}16.0 \times 10^9/\text{L}$) were determined by the automated haematology analyser (BC-2800 Vet haematology analyser Mindray Bio-Medical Electronics Co., Ltd. Nanshan, Shenzhen). Serum samples were obtained by centrifuging for 10 minutes at 3000 rpm the no anticoagulant blood sample in sterile plastic tubes. These samples were stored at -80°C until the biochemical analysis. CRP (Tri-Delta Phase Canine CRP, Tri-Delta Diagnostic, Boonton Township, NJ), suPAR (Canine suPAR ELISA Kit, Yehua Biological Technology Co. Ltd, Shanghai) and neopterin (Canine Neopterin ELISA Kit, Yehua Biological Technology Co. Ltd, Shanghai) levels of the serum samples were determined in the ELISA reader (Thermo Multiskan GO Microplate Spectrophotometer, Waltham, Massachusetts) by using commercial kits by the sandwich enzyme immunoassay method. The intra and inter assay coefficient of variation of CRP was reported by the manufacturer as 6.5-6.9% and 7.8-8.2%, respectively. The assay manufacturer reported that the assay range of suPAR was 5-2000 ng/L, sensitivity 2.43 ng/L, and intra and inter assay precision coefficient of variation $<8\%$ and $<10\%$, respectively. The measurement limit of the neopterin assay is 0.2-60 nmol/ml, sensitivity 0.11 nmol/mL, intra and inter assay coefficient of variation $<10\%$ and $<12\%$, respectively. The study protocol was approved by the Ethics Committee of Sivas Cumhuriyet University, Turkey (Approval No: 2016/36).

Statistical Analysis

The data were analyzed by using statistical package for the social science-15 (SPSS-15). Analysis of data from 45 dogs randomly selected among patient dogs, as identified by clinical and laboratory methods, and healthy dogs, revealed a statistical significant difference between the groups. The data were presented as mean and standard error of the mean (SEM). The data distribution was evaluated by Kolmogorov-Smirnov test. Comparisons between groups were made by conducting T test for continuous variables with a normal distribution, whilst variables with non-normal distribution were analyzed with the Mann-Whitney U test. Receiver operating characteristic (ROC) curves and the area under these curves were used to assess the diagnostic potential of CRP, suPAR and neopterin levels. ROC analyses were performed for both the dogs with SIRS and the healthy dogs. To assess the diagnostic potential of CRP, suPAR and neopterin levels in the diagnosis of SIRS, the area under curve (AUC) and some cut-off values were analyzed. The results were assessed at a 95% confidence interval and at a significance level of $P < 0.05$.

RESULTS

30 dogs with SIRS clinically inspected in detail and 15 healthy dogs were included in the study. Dogs < 6 months old were not included in the study. Diseases causing SIRS include: gastrointestinal ($n = 14$), genital system ($n = 4$), malignant ($n = 1$), respiratory ($n = 7$), and dermatological ($n = 4$) diseases.

Table 1 presents changes in mean values of CRP, suPAR and neopterin levels of both dogs with SIRS and the healthy, and the significance in intergroup differences. In Figure 2-4, distribution of CRP, neopterin and suPAR among dogs with SIRS and healthy are presented. It was determined that the CRP, suPAR and neopterin concentrations of dogs with SIRS were significantly higher ($P < 0.001$, $P < 0.01$, $P < 0.01$, respectively) than those of the dogs in the healthy group (Table 1).

ROC analysis was conducted on the CRP, suPAR and neopterin levels in the healthy animals and the dogs with SIRS. The ROC curves used to assess the diagnostic potential of CRP, suPAR and neopterin levels, are presented in Figure 1 and AUC are shown in Table 2. The ROC analysis was used to establish the cut-off, sensitivity and specificity values. Table 2 shows the cut-off, sensitivity, specificity and AUC values of the CRP, suPAR and neopterin levels of

healthy dogs and dogs with SIRS. The cut-off values for the CRP, suPAR and neopterin levels were determined as 11.20 mg/L, 223.68 mg/L and 3.23 mmol/mL, respectively.

Based on ROC analysis, the area below the reference line is 0.99 for CRP, 0.69 for suPAR and 0.72 for neopterin, and that these biomarkers can offer significant diagnostic information.

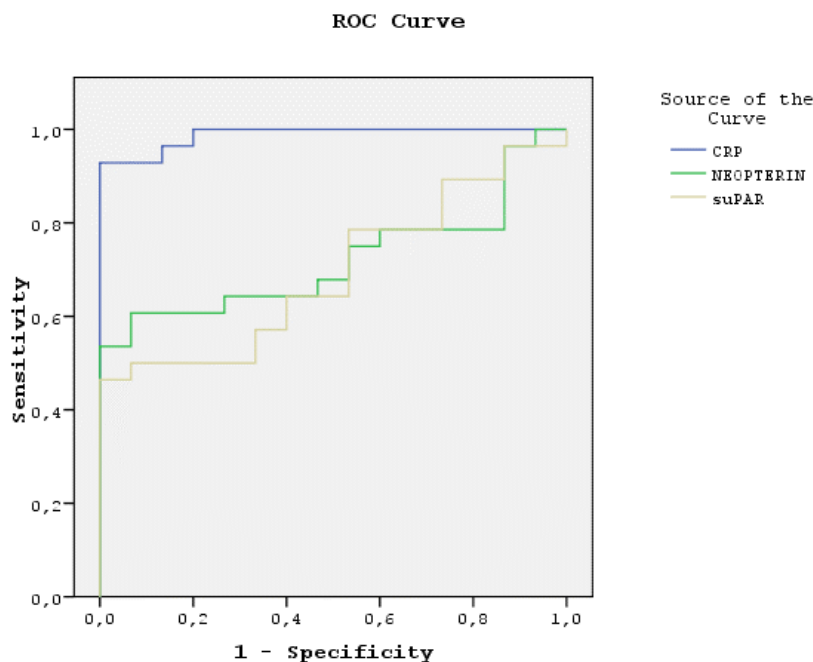


Figure 1. Receiver operating characteristic curve in the diagnosis of dogs with SIRS and healthy of serum levels CRP, suPAR and neopterin

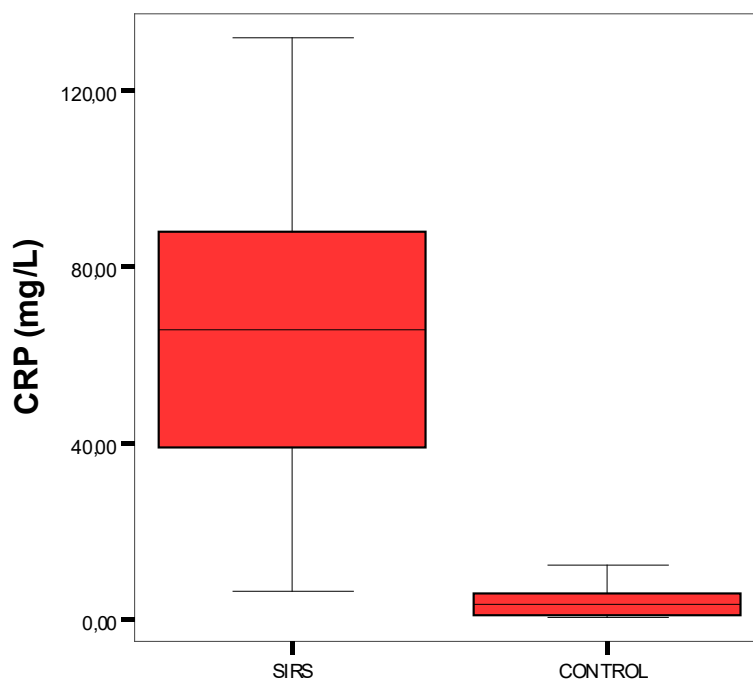


Figure 2. CRP levels in dogs with the systemic inflammatory response syndrome (n=30) and healthy (n=15)

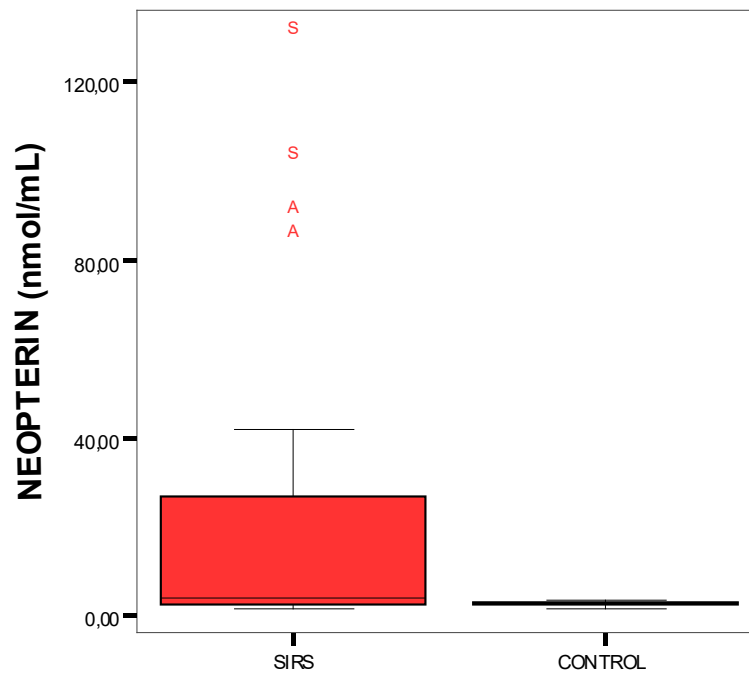


Figure 3. Neopterin levels in dogs with the systemic inflammatory response syndrome (n=30) and healthy (n=15)

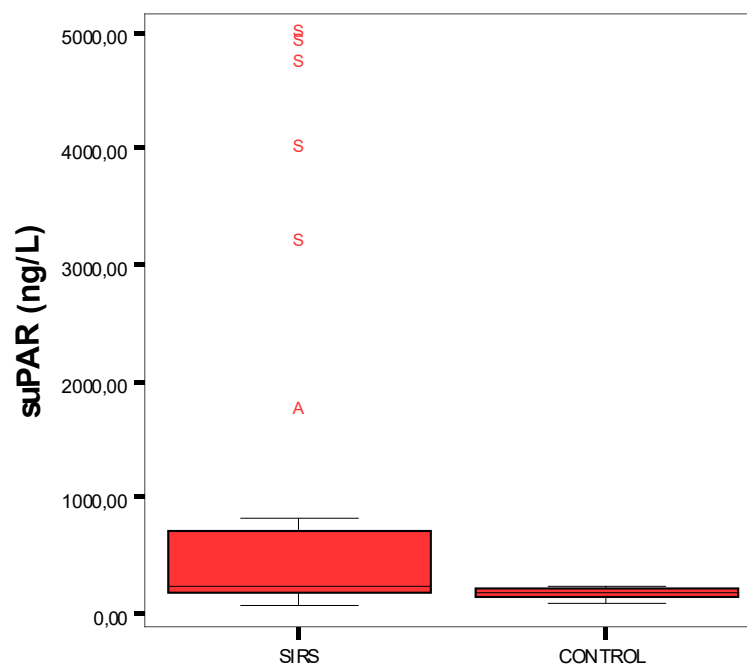


Figure 4. suPAR levels in dogs with the systemic inflammatory response syndrome (n=30) and healthy (n=15)

Table 1. The CRP, suPAR, and neopterin levels of healthy dogs and dogs with SIRS (Mean±SEM)

Parameters	Healthy group	SIRS	P value
CRP (mg/L)	4.30±0.95	66.59±6.57	< 0.001
suPAR (ng/L)	172.12 ±11.78	1001.79±287.60	0.007
Neopterin (nmol/mL)	2.63±0.15	21.08±6.36	0.007

CRP; C-reactive protein, suPAR; soluble urokinase-type plasminogen activator receptor

Table 2. The cut-off, sensitivity, specificity and AUC values of the CRP, suPAR, and neopterin levels of healthy dogs and dogs with SIRS.

Parameters	CRP (mg/L)	suPAR (ng/L)	Neopterin (nmol/mL)
AUC	0.99	0.69	0.72
Cut off	11.20	223.68	3.23
Sensitivity (%)	92.90	53.30	63.30
Specificity (%)	93.30	93.30	93.30
P value	<0.001	0.039	0.018
SEM	0.011	0.079	0.077

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

DISCUSSION

SIRS, which develops in various diseases in dogs, requires emergency treatment. Thus, urgent and correct diagnosis is critical. CRP is an acute phase protein released during tissue damage and inflammation. It is synthesized primarily in liver in response to stimulation by cytokines and is defined as major acute phase protein identified in dogs. (Jitpean et al., 2014). Serum CRP level in healthy dogs (Torrente et al., 2015) is < 35 mg/L (when analysis using LifeAssays magnetic permeability-based immunoassay), and it has been reported by various studies that it increases as a response to inflammatory stimulus (Viitanen et al., 2014). Experimental studies have reported that CRP increases before the formation of clinical symptoms and reaches peak levels after 36 hours (Spapen et al., 2006). It has also been reported that CRP increases in diseases such as leptospirosis, babesiosis, parvovirus infections, malignant tumors, pyometra, acute pancreatitis, immune hemolytic anemia, arthritis, glomerulonephritis (Holm et al., 2004; Matijatko et al., 2007; Nakamura et al., 2008; Kocaturk et al., 2010; Tostes Oliveira et al., 2010). In a study on dogs with monocytic ehrlichiosis, it has been found that CRP is an indicator in determining the clinical phase of the diseases, however, is not useful in determining the clinical result (Mylonakis et al., 2011). It was determined that high CRP levels in dogs with pyometra increased the rate of mortality and extended the treatment duration. CRP levels in dogs with no systemic inflammation ranged between 0.07-24.7 mg/L, and that CRP levels in dogs with systemic inflammation ranged between 0.4-907.4 mg/L (Christensen et al., 2014). In this study, it was determined that average CRP levels of dogs with SIRS were significantly higher ($P < 0.001$) than those of healthy dogs. Besides, the CRP cut-off value was considered to be 11.20 mg/L for the healthy dogs and the dogs with SIRS with sensitivity 92.9%, and specificity 93.3%. In our study, it was observed

that CRP has high sensitivity and specificity for the detection of systemic inflammatory response in dogs and can be evaluated as a good biomarker of inflammation in dogs similar to other studies.

Biochemical markers are used to predict the morbidity and mortality as well as the diagnosis of the pathological condition. In veterinary medicine, biochemical parameters have been investigated for diagnostic and prognostic purposes in dogs with SIRS and sepsis (Kocaturk et al., 2010; Torrente et al., 2015; Ok et al., 2015). Inflammatory mediators showing immune activation such as suPAR and neopterin are investigated in order to provide information on clinical diagnosis and prognosis of infectious diseases in human medicine (Murr et al., 2002; Eugen-Olsen 2011; Zeng et al., 2016). suPAR is a marker of fibrinolysis and inflammation that is released from immunologically active cells. suPAR reveals the patho-physiological mechanisms that are active on cell surface (Wittenhagen et al., 2004; Kofoed et al., 2008; Kuleš et al., 2017). In a study comparing dogs with babesiosis and healthy ones, significant increases in suPAR levels were determined and it was stated that it would be useful in the evaluation of the stage of the disease. It was also reported that increased suPAR concentrations may be a reflection of inflammatory response in dogs with babesiosis (Kuleš et al., 2017). In a study on patients with sepsis in intensive care, it was stated that there is an increase in suPAR level throughout acute inflammation (Kofoed et al., 2008). In another study, blood suPAR was shown to be significantly increased in patients with sepsis and stated that it was a valuable parameter to differentiate SIRS from sepsis. They also reported that the use of suPAR and procalcitonin (PCT) combination increases the diagnostic efficacy for sepsis in human medicine (Zeng et al., 2016). In other studies, it was found that suPAR increased significantly in people with SIRS and sepsis, but it

was concluded that the diagnostic value of suPAR in sepsis was insufficient according to classical markers such as PCT and CRP. The reason for this is that the strong induction of PCT and CRP with lipopolysaccharides may explain its benefits in the diagnosis of bacterial infection, whereas suPAR is induced at a lower level with bacterial proteins such as lipopolysaccharide. Considering all these results, suPAR has limited diagnostic value in sepsis compared to classical markers such as PCT and CRP (Kofoed et al., 2007; Koch et al., 2011; Hoenigl et al., 2013; Bilgili and Cinel 2013). The study revealed that the average suPAR levels of dogs with SIRS were significantly higher ($P < 0.01$) than those of healthy dogs. The cut-off value of suPAR was considered as 223.68 ng/L for healthy dogs and dogs with SIRS, its sensitivity was determined to be low (53.3%), and its specificity as high (93.3%). In our study, it was found that suPAR had low sensitivity compared to CRP, which is a good marker for the detection of inflammation in dogs. The results of our study show that suPAR is insufficient for the diagnosis of systemic inflammatory response in dogs similar to the studies in human medicine.

Neopterin is a critical indicator for cellular immune system activation released by monocytes and macrophages (Hoffmann et al., 2003). It was determined that in intensive care units neopterin levels of patients with sepsis, septic shock, SIRS, or with multiple organ failure increased (Berdowska and Zwirska-Korczala 2001; Alberti et al., 2003; Baydar et al., 2009). It was reported that the neopterin level increased in viral, bacterial, and parasitic infections, apart from sepsis (Berdowska and Zwirska-Korczala 2001; Murr et al., 2002). It was stated that the neopterin levels increased in neonatal calves with septicemic colibacillosis, in relation to IFN- γ as a defense mechanism (Ercan et al., 2016). In a study conducted on 208 intensive care patients in human medicine, the cut-off value of the recommended neopterin for the diagnosis of infection was 18 pg/L (Brunkhorst et al., 1999). In the study, it was found that the se-

rum neopterin levels of dogs with SIRS was higher in comparison to the healthy dogs and that this increase is statistically significant ($P < 0.01$). The cut-off value of neopterin was considered as 3.23 nmol/mL for SIRS, with sensitivity 63.3%, and specificity 93.3%. In this study, the sensitivity of neopterin was found to be lower than CRP, although higher than suPAR. In human medicine (Brunkhorst et al., 1999), neopterin has been reported to be more sensitive than classical markers of inflammation (such as procalcitonin). On the contrary, in this study, it was observed that systemic inflammation in dogs is less sensitive than CRP, one of the classical biomarkers.

CONCLUSIONS

It was supported by the comparative ROC analysis that CRP has more diagnostic significance than neopterin and suPAR. In the ROC analysis, it was determined that CRP had the highest sensitivity, followed by neopterin and suPAR, respectively, and that CRP, suPAR and neopterin had the same values in terms of specificity. As a result, we determined that CRP showed the best performance for detection of systemic inflammatory response in dogs, and neopterin and suPAR could be used as an alternative to CRP. It is also concluded that further studies are necessary in order to determine diagnosis and prognosis of diseases.

ACKNOWLEDGEMENTS

Supported by the Coordinator of Scientific Research Projects of Sivas Cumhuriyet University (Project number: V-040).

Presented in part in abstract form at the I. International Turkish Veterinary Internal Medicine Congress, Antalya, Turkey, October 10-13, 2017.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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