

Quantitative and Qualitative Urine Protein Excretion in Dogs with Severe Inflammatory Response Syndrome

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Background: Proteinuria is an established characteristic of renal disease in dogs, providing diagnostic and prognostic information. Little is known about the occurrence and severity of proteinuria in dogs with severe inflammatory response syndrome (SIRS).

Hypothesis: The quantitative and qualitative urinary protein (UP) excretion is altered in dogs with SIRS.

Animals: Thirty-nine dogs with SIRS and 15 healthy control dogs at admission.

Methods: A case control study was performed. Diagnosis of SIRS was based on clinical and clinicopathological findings. Urinary protein (UP) was measured by a colorimetric assay. Urinary albumin (UAib) and urinary retinol-binding protein (URBP) were measured by ELISA and quantified in relation to urinary creatinine (UC). Sodium dodecyl sulfate polyacrylamid-gel electrophoresis was conducted to identify the qualitative pattern of proteinuria. Mann–Whitney *U*-test was used to assess differences in UP/UC, UAib/UC and URBP/UC between the groups. *P*-values < .05 were considered significant.

Results: Dogs with SIRS had higher ratios of UP/UC, UAib/UC and URBP/UC (all *P* < .001) in comparison to healthy control dogs. Dogs with SIRS had a total of 11 protein bands compared to 3 bands in healthy controls. In dogs with SIRS, 58% of the total counted bands were in the low molecular weight range (<60 kDa) whereas 42% were in the middle (60–80 kDa)/high molecular weight range (>80 kDa).

Conclusions and Clinical Importance: SIRS alters UP excretion in dogs. Further studies should evaluate whether or not the magnitude of proteinuria is predictive of the severity and outcome of dogs with SIRS.

Key words: Albumin; Inflammation; Proteinuria; Retinol-binding protein.

Severe inflammatory response syndrome (SIRS) and sepsis pose serious risks for critically ill dogs.^{1,2} Studies have shown case fatality of up to 30–50% for dogs with SIRS.^{2–5} Human research has demonstrated that massive production of proinflammatory cytokines and activation of the coagulation cascade are characteristics of SIRS and sepsis.^{6,7} Subsequent microvascular thrombosis and endothelial cell damage increase capillary permeability, enhance the inflammatory state, and trigger tissue ischemia and organ failure.^{6,7} In the kidneys the resulting capillary leak leads to increased urinary excretion of plasma proteins.^{8,9} Acute inflammation accompanying trauma, surgery, or ischemia in humans is associated with the rapid onset of microalbuminuria.^{8,10} Consequently the increased excretion of protein in urine was investigated as a biomarker for SIRS in human patients.^{8,10,11} Albuminuria was recorded in 72% of dogs admitted to an intensive care unit (ICU). This group included a small number of dogs with inflammation, sepsis, or immune-mediated hemolytic anemia (IMHA), all of which demonstrated abnormal amounts of urine albumin. In general, there

Abbreviations:

HMW	high molecular weight
IMHA	immune-mediated hemolytic anemia
LMW	low molecular weight
MMW	middle molecular weight
SDS-PAGE	sodium dodecyl sulfate polyacrylamid-gel electrophoresis
SIRS	severe inflammatory response syndrome
UAib	urinary albumin
UC	urinary creatinine
UP	urinary protein
URBP	urinary-retinol-binding protein

is a higher prevalence of albuminuria in dogs that are euthanized or die within 3 days of admission to the ICU as compared to dogs who survive more than 3 days.¹²

Under physiological circumstances, the renal processes of glomerular filtration, tubular reabsorption, and tubular secretion regulate the quantity of protein in urine of dogs.¹³ The qualitative protein excretion in dogs is characterized by specific proteinuric patterns that can reflect renal injury at various sites of the nephron.^{14,15} For instance, glomerular proteinuria occurs when the selective permeability of the glomerular filtration barrier is altered and thereby leads to filtration of molecules whose size or combined size and charge would normally prevent this. In this case, the level of proteins in the urine is usually quite high and the proteinuria is characterized by the presence of middle molecular weight (MMW; approximately 60–80 kDa) to high molecular weight (HMW; >80 kDa) proteins.^{14,15} The presence of only MMW proteins in urine, such as albumin, is defined as “selective” glomerular proteinuria, whereas HMW proteins, such as IgG,

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indicate “nonselective” glomerular proteinuria.¹⁵ Tubular proteinuria on the other hand is characterized by the presence of low molecular weight (LMW; <60 kDa) proteins, such as retinol-binding protein (RBP), which would normally freely pass the glomerular filtration barrier but should be reabsorbed in the proximal tubule.^{16,17} When both glomerular filtration and tubular reabsorption are disturbed, mixed proteinuria can occur. An underlying cause for this is often the glomerular filtration of abnormally high amounts of proteins so that the competitive affiliation for the tubular reabsorption by the megalin-cubilin complex reaches a saturation point.¹⁸ In this case, LMW, MMW, and HMW proteins are present in the end urine.^{15,18,19}

Although the early recognition of SIRS in dogs has received increasing attention in recent years,^{3–5} the occurrence of proteinuria within the framework of acute inflammatory response in dogs has received little attention. Therefore, this study was performed to investigate the occurrence and severity of proteinuria in dogs with SIRS. Besides the quantification of the total protein content, the urinary proteins were qualitatively investigated by assessing the protein pattern as well as the presence of albumin, a known marker of glomerular injury, and RBP, as a sensitive marker of proximal tubular dysfunction.¹⁶

Material and Methods

Patient and Control Groups

All dogs for this study were presented at the Small Animal Clinic at the Freie Universität Berlin between April 2004 and October 2005. Dogs in the study group demonstrated 2 determining factors, namely (1) they were presented with an acute and serious clinical condition and (2) following examination, they fulfilled the criteria for SIRS in dogs (Table 1).^{1,2} Standard diagnostic methods were used to investigate the underlying conditions. The diagnosis of primary IMHA was based on the occurrence of anemia, a positive direct Coombs' test, or persistent autoagglutination of erythrocytes and numerous spherocytes, plus the exclusion of underlying diseases or triggering factors.^{20,21} All dogs were treated according to standard practice for emergency patients. Routine biochemical and hematological blood tests were performed for each dog.^{5,21} None of the dogs diagnosed with IMHA had received glucocorticoid treatment. Dogs for the control group were chosen randomly from animals presented at the clinic for the purpose of blood donation or routine examination. The control dogs were examined once on presentation and based on history, clinical, hematological, biochemical, and urinary examinations, these dogs were deemed to have had neither previous history nor any current clinical signs of renal impairment.

Table 1. Inclusion criteria for SIRS.^{1,2}

SIRS = ≥ 2 of the Following Criteria	
Hypo- or hyperthermia (°C)	<37.8 or >39.4
Tachycardia (heart rate [beats/min])	>140
Tachypnea (respiratory rate [breaths/min])	>20
Leukopenia or leukocytosis (WBC/ μ L)	<6,000 or >16,000
Immature (band) neutrophils (%)	>3

SIRS, severe inflammatory response syndrome; WBC, white blood cells.

Urine Sampling

Urine was collected from each dog at the time of admission. The urine samples were collected by means of midstream catch of voluntary urination, cystocentesis, or catheterization. Samples with macroscopic hematuria were excluded from the study. Urine was centrifuged for 2 minutes at $300 \times g$ to remove cells and particulate matter. The urine samples were kept frozen at -80°C until use; all assays were performed ≤ 4 months after collection.

Quantitative Analysis of Proteinuria

The concentrations of urinary protein (UP) and urinary creatinine (UC) were measured colorimetrically in a microtiter plate reader^a by using commercially available kits.^{b,c} The concentrations of urinary retinol-binding protein (URBP) and urinary albumin (UAlb) were determined by using established sandwich ELISA techniques.^{16,22} For URBP measurement, polyclonal rabbit-antihuman RBP^d was used as catching antibody and a cross-reacting peroxidase-conjugated rabbit antihuman RBP^e was used as detection antibody. Cross-reactivity of the antihuman RBP with dog RBP was tested by use of western blot analysis, as described elsewhere.^{23,24} For UAlb measurement, polyclonal goat antidog albumin^f IgG was used as catching antibody and a peroxidase-conjugated IgG fraction of goat antidog albumin^g was used as detection antibody. In both URBP and UAlb ELISAs, a citric acid solution of o-phenylenediamine dihydrochloride^h with 0.012% hydrogen peroxide was used to develop the substrate reaction. The reaction was stopped by addition of 1 mol/L H_2SO_4 and the absorbance of each well was measured by use of a microtiter plate reader.^a The standard curve obtained for each plate was used to calculate URBP and UAlb concentrations in the samples on that plate. The intra-assay and interassay coefficients of variation (CVs) for URBP were 2.9 and 4.2%, respectively. Intra- and interassay CVs for UAlb were 8.0 and 9.2%, respectively. The UP/UC, URBP/UC, and UAlb/UC were calculated for each dog so as to negate differences in absolute protein excretion attributable to varying urine concentrations.

Qualitative Analysis of Proteinuria

Initially, a 10% sodium dodecyl sulfate polyacrylamid-gel electrophoresis (SDS-PAGE) of each urine sample (5 μ L aliquots) and of standard proteinsⁱ was performed with a minigel apparatus.^j The separated proteins were stained with the sensitive silver nitrate method²⁵ and the gels were examined with an imager^k and software^l to assess the molecular weights of the urinary proteins that classify proteinuric patterns.^{15,19} The SDS-PAGE pattern characterized by the presence of a 66 kDa band (albumin) alone was defined as physiological proteinuria. The pattern characterized by the presence of proteins below 66 kDa was named as LMW proteins of tubular origin. The proteins with molecular weights between 66 and 80 kDa were named as MMW proteins, and the proteins with a molecular weight above 80 kDa were named as HMW proteins of glomerular origin. The patterns characterized by the presence of HMW, MMW, and LMW proteins were defined as mixed origin.²⁶

Statistical Evaluation

Results were expressed as medians (50th percentile) and interquartile ranges (Q1 is the 25th percentile and Q3 is the 75th percentile). The Kolmogorov–Smirnov-test^m was used to confirm

that the data were not normally distributed. The differences between the groups were analyzed by using the Mann–Whitney *U*-test for nonparametric data.^m Spearman rank correlation coefficients^m were used to test the association between variables used to evaluate proteinuria. Differences were considered significant at a *P* value of $\leq .05$.

Results

Study and Control Groups

The group with SIRS comprised 39 dogs with a median age of 8 years (Q1 = 6; Q3 = 10; range: 1–13 years) of which there were 18 intact and 2 spayed bitches, 15 intact and 4 neutered males. The most frequently represented breeds were German Shepherd ($n = 6$) and mixed breed ($n = 6$), and in total, 21 different breeds were represented. Weights ranged from 8.0 to 50.0 kg with a median of 29.0 kg (Q1 = 15.0; Q3 = 37.0). The dogs were presented for a variety of underlying conditions as listed in Table 2. The control group comprised 15 dogs with a median age of 4 years (Q1 = 3; Q3 = 5; range: 1–10 years). It included 3 intact and 3 spayed bitches, 3 intact and 6 neutered males, and dogs were predominantly mixed breed. The median weight of the control dogs was 24.5 kg (Q1 = 10.6; Q3 = 33.0; range: 5.9–36.2 kg).

Table 2. Underlying diseases for the group of SIRS affected dogs ($n = 39$).

Underlying Disease	Number of Dogs
Primary immune-mediated hemolytic anemia	11
Pyometra/hemometra	8
Peritonitis (septic/nonseptic)	6
Abscesses	7
Fever of unknown origin	2
Necrotizing tonsillitis	1
Parvovirus	1
Salmonellosis	1
Splenic neoplasia	1
Underlying disease undiagnosed	1

SIRS, severe inflammatory response syndrome.

Quantitative Analysis of Proteinuria

Based on the International Renal Interest Society (IRIS) guidelines²⁷ from 2006, 27 dogs (69%) from the SIRS group had proteinuria with a value of UP/UC in excess of 0.5. Of these dogs, 11 had a UP/UC between 0.5 and 1.0 and 16 dogs had overt proteinuria as indicated by a value of UP/UC exceeding 1.0 (5 dogs) and 2.0 (11 dogs). Five SIRS dogs and 1 healthy control dog had a value of UP/UC in the borderline proteinuric range of 0.2–0.5,²⁷ whereas the remaining 7 SIRS dogs and 14 healthy controls all had a value of UP/UC below 0.2. Therefore, the UP/UC for dogs with SIRS (median: 0.65; Q1 = 0.37; Q3 = 2.41; range: 0.09–7.94) was significantly higher ($P < .001$) than that for healthy controls (median: 0.09; Q1 = 0.06; Q3 = 0.10; range: 0.03–0.34; Fig 1).

Dogs with SIRS also exhibited significantly higher ($P < .001$) UAlb excretion as expressed by the UAlb/UC ($\mu\text{g}/\text{mg}$) ratio (median: 46.1; Q1 = 15.2; Q3 = 123; range: 3.1–1,338) in comparison with the healthy group (median: 1.9; Q1 = 0.35; Q3 = 3.65; range: 0.2–8.3; Fig. 1). When considering microalbuminuria as an UAlb/UC ratio $>30 \mu\text{g}/\text{mg}$,²⁸ 25 (64%) of the SIRS dogs had microalbuminuria on the day of admission. Five dogs exhibited UAlb/UC levels above 300 $\mu\text{g}/\text{mg}$ and extrapolating from human studies, could therefore be described as having overt albuminuria.¹¹ In contrast, all 15 healthy dogs had UAlb/UC levels below 30 $\mu\text{g}/\text{mg}$. The levels of URBP/UC ($\mu\text{g}/\text{mg}$) were also significantly higher ($P < .001$) in dogs with SIRS (median: 0.11; Q1 = 0.07; Q3 = 0.17; range: 0.02–0.79) compared with the control group (median: 0.018; Q1 = 0.01; Q3 = 0.02; range: 0.009–0.03; Fig 1). By using data of all dogs investigated, the Spearman's rank correlation test showed strong positive correlations between the UP/UC and UAlb/UC ($\rho = 0.776$), UP/UC to URBP/UC ($\rho = 0.576$) and UAlb/UC to URBP/UC ($\rho = 0.583$). In each case the *P* value was $< .001$.

Qualitative Analysis of Proteinuria

An SDS-PAGE of urine samples was conducted for each SIRS and control dog. Figure 2 shows the

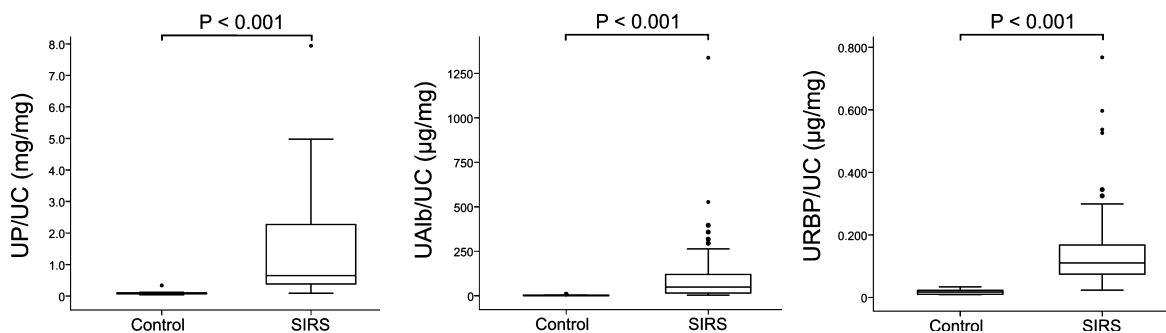


Fig 1. Box plots of urinary protein-to-urinary creatinine ratio (UP/UC), Urinary albumin-to-urinary creatinine ratio (UAlb/UC) and urinary retinol-binding protein-to-urinary creatinine ratio (URBP/UC) in control dogs and dogs with severe inflammatory response syndrome (SIRS). The box represents the interquartile range (ie, 25–75% range); the horizontal bar represents the median value, and the T-bars represent the range of the data; outliers are solitary points outside whiskers.

molecular weight ranges and the frequency of appearance of the protein bands in each molecular weight range. A UP band in the range of 60–80 kDa dominated in the control dogs. Three control dogs, all sexually intact males, further exhibited protein bands below 30 kDa. In healthy dogs, 55% of counted bands were in the MMW/HMW range and 45% were of the LMW type. The electrophoresis patterns in urine from the SIRS dogs were far more heterogenic and a total of 11 different bands were observed (Fig 3). As with the healthy dogs though, the presence of a band in the range 60–80 kDa predominated, occurring in 36 (92%) dogs. A further pattern was evident as 23 of these SIRS dogs also exhibited a 90–100 kDa protein. The distribution of the numbers of bands in the categories MMW/HMW and LMW was different from that of the healthy animals in that 42% of the observed proteins were in the MMW/HMW range and 58% in the LMW range. Based on the presence of bands in the

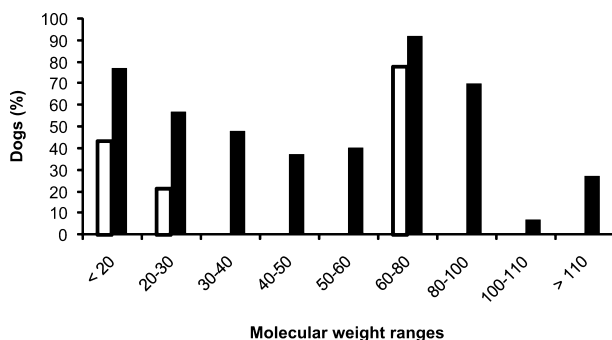


Fig 2. Frequency of dogs that had bands of each molecular weight range, as detected by sodium dodecyl sulfate polyacrylamid-gel electrophoresis analysis. White bars indicate the control dogs and black bars indicate dogs with severe inflammatory response syndrome.

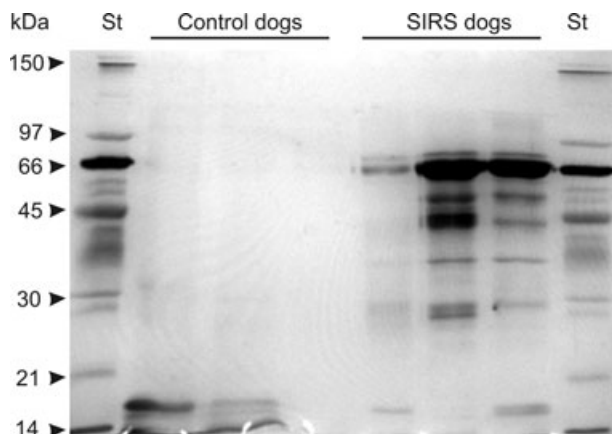


Fig 3. Silver nitrate stained sodium dodecyl sulfate polyacrylamid-gel electrophoresis of urine samples from the healthy control dogs and dogs with severe inflammatory response syndrome (SIRS). Vertical lanes contain the standard (St) and urine from representative dogs.

various molecular weight categories in the SDS-PAGE,¹⁴ the protein pattern of each diseased dog was interpreted as physiological, glomerular, tubular, or of mixed nature as shown in Table 3.

Discussion

This study showed that proteinuria is present in dogs with SIRS as indicated by significantly higher ratios of UP/UC, UAlb/UC, and URBP/UC compared to healthy control dogs. From the SIRS group, 27 dogs (69%) exceeded the upper normal limit for the UP/UC ratio of 0.5 and 25 dogs (64%) demonstrated UAlb/UC levels higher than the currently used upper norm of 30 $\mu\text{g}/\text{mg}$ for detection of microalbuminuria in dogs.^{29–31} Systemic capillary leak and an increased protein permeability of the glomerular filtration membrane caused by SIRS might be a possible explanation for these findings.^{9,10,32} In comparison, all control dogs had UP/UC and UAlb/UC ratios within the suggested reference ranges.

In respect to the URBP excretion, there are currently no reference values for dogs available, necessitating the definition of upper normal levels for URBP and URBP/UC. Based on the mean value from the healthy controls plus 2 times the standard deviation (mean + 2 SD) as described previously,³³ the upper limit for normal URBP excretion was defined as 60.2 $\mu\text{g}/\text{L}$ and 0.033 $\mu\text{g}/\text{mg}$ for the URBP/UC. By using these reference values, the URBP content and the URBP/UC levels were above normal in 37 (95%) and 36 diseased dogs (92%), respectively. One healthy dog exceeded the upper limit for URBP/UC marginally (0.034 $\mu\text{g}/\text{mg}$). A recent study¹⁶ on 8 healthy dogs (defined as “healthy” by a GFR > 90 mL/min/m² body surface area and a value of UP/UC < 0.2) recorded a median URBP/UC of 0.01 $\mu\text{g}/\text{mg}$ (range of 0.007–0.03 $\mu\text{g}/\text{mg}$), which is comparable to values from healthy dogs in this study (median 0.018 $\mu\text{g}/\text{mg}$, range 0.009–0.034 $\mu\text{g}/\text{mg}$).

The presence of RBP in the end urine is possibly not just the result of saturation of the tubular reabsorption mechanisms with albumin and other MMW/HMW proteins and their competition for receptor-binding sites,¹⁸ but could also be because of direct tubular damage induced by SIRS. Several experimental studies on rats have explored this possibility.^{34,35} One study³⁴ investigated the effects of inflammatory cytokines on tissue cultures of rat proximal tubular epithelial cells, and demonstrated that exposure of tubular epithelial cells to TNF- α caused a decrease in the expression of megalin, the most important receptor for re-uptake of LMW proteins in the renal tubules.³⁶ In addition to this and of relevance for SIRS, TNF- α and TGF- β_1 also appeared to have differing effects on the activation of plasminogen and therefore on fibrinolysis, which is of consequence for the formation of microthrombi and ensuing tissue hypoxia.³⁴ Another study³⁵ investigating the effect of ischemia-reperfusion injury on specific sodium transporters on the apical membrane of the renal tubule showed both their

Table 3. Total number and percentage of control and SIRS dogs with each type of proteinuria, based on optical assessment of SDS-PAGE.

	n	Physiological Proteinuria (%)	Glomerular Proteinuria (%)	Tubular Proteinuria (%)	Mixed Proteinuria (%)
Control dogs	15	15 (100)	n/d	n/d	n/d
SIRS dogs	39	8 (21)	2 (5)	11 (28)	18 (46)

n/d, not detectable; SDS-PAGE, sodium dodecylsulfate polyacrylamid gel electrophoresis; SIRS, severe inflammatory response syndrome.

expression and activity to be greatly reduced. A recent human study³⁷ also examined URBP excretion in critically ill patients and found increased levels to be highly indicative of acute renal injury. Interestingly, the vast majority of diseased dogs in the present study (36 dogs, 92%) had increased URBP/UC, regardless of whether their UP/UC or UAlb/UC levels were affected. Therefore, the high level of URBP/UC in the diseased dogs is suggestive of a SIRS-induced impairment of tubular function.

The electrophoresis patterns of the healthy dogs in this study appeared representative of normal urine samples, and similar results have been obtained by other authors.^{14,15,38} In the current study, where in total 3 different bands were observed in urine of healthy dogs, albumin was the most predominant, albeit faintly stained band. It has been reported that dogs without impairment of kidney function regularly show an albumin band, which is considered physiological providing the animal's UP/UC ratio is below 0.5.¹⁴ This was the case for all healthy control dogs. The 2 faintly stained LMW bands, which appeared in electrophoresis from 3 sexually intact healthy males, were likely prostate-specific proteins, which reportedly occur regularly in urine of healthy entire males.^{39,40} It has been shown, however, that the total amount of UP does not differ between healthy entire or neutered males.³⁹

Besides the quantitative analysis of URBP/UC, the presence of tubular lesions was further emphasized in the SDS-PAGE through the high numbers of SIRS dogs with bands in the LMW ranges below 66 kDa. Dogs with pyometra have a high prevalence of LMW bands, even to the point that some LMW proteins in the 30–60 kDa range are found only in the urine of these dogs but not in healthy controls.³⁸ A similar situation was observed in SDS-PAGE samples in the present study, where only 45% of bands from healthy controls were LMW bands as opposed to 58% of bands in dogs with SIRS. The abundance of LMW proteins observed in the SDS-PAGE from SIRS dogs, regardless of sex, most probably masked any subtle sex differences as observed in the healthy dogs. In regard to proteinuria of glomerular origin, three SIRS dogs did show a discrepancy between their above-normal UAlb/UC and the SDS-PAGE interpretation of physiological proteinuria (1 dog) and tubular proteinuria (2 dogs). The possible fragmentation of albumin into a 30 kDa breakdown product, which has been reported in humans,^{41,42} could be a possible explanation for the observed discrepancy; however, there is

currently no information about protein degradation within the tubular lumen in dogs.

In conclusion, this study showed that proteinuria is a common occurrence in dogs identified as having SIRS and that the increased levels of UP/UC, UAlb/UC, and URBP/UC were an indication that elements of both glomerular and tubular malfunction were present. Further studies should evaluate whether or not the magnitude of proteinuria is predictive of the severity and outcome of SIRS patients.

Footnotes

^a Microplate reader Model 680 XR, Bio-Rad, Munich, Germany

^b Bradford protein assay, Bio-Rad

^c Creatinine assay, Randox Laboratories, Crumlin, UK

^d Code A0040, DakoCytomation, Hamburg, Germany

^e Code A0304, DakoCytomation

^f Code A40-113A, Bethyl Laboratories Inc, Montgomery, TX

^g Code A40-113P, Bethyl Laboratories Inc

^h Sigma-Aldrich, Taufkirchen, Germany

ⁱ LMW range protein standard, Bio-Rad

^j Mini Protean System, Bio-Rad

^k ChemiDoc XRS scanner system, Bio-Rad

^l Quantity One software, Bio-Rad

^m SPSS, version 11.0, SPSS Inc, Munich, Germany

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