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Investigations on the quantitative and qualitative protein excretion in urine of dogs with Severe Inflammatory Response Syndrome (SIRS)

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List of abbreviations

DBP	Vitamin D-binding protein
DIC	Disseminated intravascular coagulation
GAGs	Glycosaminoglycans
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
HDL	High density lipoprotein
HMW	High molecular weight proteins
LMW	Low molecular weight proteins
MMW	Medium molecular weight proteins
IFN-γ	Interferon gamma
IMHA	Immune mediated haemolytic anaemia
IL	Interleukin
MA	Microalbuminuria
MODS	Multiple organ dysfunction syndrome
PGE-2	Prostaglandin E-2
RBP	Retinol-binding protein
SDS-PAGE	Sodiumdodecyl-sulfat-polyacrylamid-gel electrophoresis
SIRS	Severe inflammatory response syndrome
TNF-α	Tumour necrosis factor alpha
UAC	Urinary albumin to urinary creatinine ratio
UPC	Urinary protein to urinary creatinine ratio
URBPC	Urinary retinol-binding protein to urinary creatinine ratio
USG	Urine specific gravity
WBC	White blood cells

1 Introduction

Proteins in the blood are subjected to the filtering and reabsorption processes of the renal glomeruli and tubules in such a way as to ensure that under physiological circumstances, the protein content in urine is quite minimal. Pathological levels of urinary protein excretion, known as proteinuria, occur when the renal mechanisms controlling protein passage are impaired. Proteinuria is classified based on the location of the pathology which leads to the presence of protein in the urine and therefore specific proteinuric patterns are characteristic for lesions in various areas of the nephron. Depending on the presence of proteins with certain molecular weights, it is possible to identify whether the renal lesions are mostly located in the glomeruli or the tubules and therefore whether filtration or reabsorption mechanisms are failing. In both dogs and humans, a variety of diseases and pathological conditions are known to cause proteinuria, the level and duration of which can be indicative of the severity of kidney damage.

Severe Inflammatory Response Syndrome (SIRS) is observed in humans affected by trauma, surgery, burns and the critically ill. Despite intensive research in recent years, this highly prevalent syndrome is still accompanied by diagnostic and therapeutical challenges and a high mortality rate. During an extreme systemic inflammatory response, the unbalanced release of pro-inflammatory and anti-inflammatory cytokines as well as the resulting response of the immune system leads to the development of SIRS. Certain cytokines which circulate at high levels during the acute phase of SIRS are believed to cause changes in capillary permeability, the effects of which can be instrumental in the development of sepsis, severe sepsis, the failure of multiple organ systems and ultimately death. In this context, SIRS has been associated with acute renal failure in human patients. In recent years a number of studies have been conducted in human medical fields to explore possible early markers for the severity of SIRS. It is believed that SIRS induced increased capillary permeability leads to a transient increase in urinary protein content so that proteinuria has been suggested as an indicator of SIRS severity in humans.

The prevalence and recognition of SIRS in critically ill canine patients has received increasing attention in recent years. Proteinuria has been reported to occur in a number of conditions in dogs and it is already widely in use as a diagnostic tool for a variety of diseases both renal and non-renal. However the occurrence of proteinuria within the framework of acute inflammatory response in dogs has received little attention. It can be hypothesized that in dogs, as with humans, SIRS will detrimentally affect the ability of the kidneys to properly maintain glomerular filtration and the reabsorption of proteins. It may also be possible to

estimate the severity of the inflammatory response based on the level of urinary protein excretion so that a mortality prognosis can be made.

The goal of this study therefore, was to investigate the occurrence and severity of proteinuria and to verify urinary biomarkers for the detection of both glomerular and tubular injury in dogs with SIRS. Furthermore the thesis aimed to elucidate whether associations can be drawn between proteinuria and mortality.

2 Literature Review

2.1 Mechanisms of proteinuria

As part of their function as molecular sieve, the kidneys play a large role in controlling the passage of plasma proteins from the blood into the ultrafiltrate and eventually into the end urine. The two basic mechanisms of kidney function, which lead to the production of urine and herewith determine the urine protein content, are glomerular filtration and tubular reabsorption. A further function also influencing urine protein content is tubular protein secretion (Schmidt and Thews, 1987; Bachmann et al., 1990; Raila et al., 2005).

2.1.1 Glomerular filtration of plasma proteins

The first step, formation of ultrafiltrate takes place in the glomerulum. This process is dependent on the structure and function of the filtration barrier, the glomerular filtration rate (GFR), blood pressure and the molecular characteristics of the plasma components (Brenner et al., 1978; Schmidt and Thews, 1987; Raila et al., 2005). The nephrons are the functional units where filtration and reabsorption takes place. The number of nephrons per kidney varies from several hundred in lower vertebrates, up to 400 000 for dogs, around a million for humans and even 4 million for cows (Liebich, 1999; Eckert, 2002). The nephrons are comprised of (1) the corpusculum renale or Malpighi body with its glomerulus, a capillary conglomerate branching off the Vasa afferentia and the Bowman capsule, a blind ending epithelial tube which engulfs the capillary conglomerate and (2) the system of renal tubules.

Separating the capillary lumen and the lumen of the Bowman's capsule is the actual glomerular filtration barrier which is comprised of three structures: (1) the fenestrated endothelium which lines the glomerular capillaries, (2) the glomerular basement membrane (GBM) made of type IV collagen, glycoproteins and a proteoglycan/sialinic acid matrix and (3) the podocytes with their interdigitating foot processes separated by filtration slits (Barber et al., 1996; Raila et al., 2005). The epithelial slit diaphragm of the Bowman capsule is made up of the foot processes of the podocytes whose GBM side cell membranes contain a transmembrane protein, nephrin (Brenner et al., 1978; Waller et al., 1989; Barber et al., 1996). Based on charge attractions, nephrin interdigitates between the slim, finger like podocyte foot processes and has therefore a vital role in maintaining an intact glomerular barrier (Ruotsalainen et al., 1999; Haraldsson and Sorensson, 2004).

The endothelium, the GBM and the slit diaphragm contain pores which decrease in size from the endothelium to the filtration slit membrane. The endothelium pores are reportedly around 70-100 nm in size and allow passage of all substances other than plasma cells and

molecules larger than 400 kDa. The slit membrane on the other hand, with pores of around 7.5 nm only allows free passage for molecules whose effective radii are less than 2 nm and have a molecular weight no larger than 40 kDa (Waller et al., 1989; Gekle, 1998). Molecules with larger radii are partly or completely retained (Waller et al., 1989).

Although the kidneys represent only about 1% of the total body weight they have an extremely high blood flow, receiving approximately 25 % of the cardiac minute volume (Lameire, 2005). This results in a huge hydraulic force in the flow of fluid across the filtering structures (Brenner et al., 1978). Due to the nature of the glomerular filtration barrier, the ultrafiltrate collected in the lumen of the Bowman's capsule is essentially iso-osmotic to the blood, containing water, electrolytes, amino acids, sugars and small protein molecules and differing to blood in regards to its lack of larger proteins and blood cells (Liebich, 1999). When regarding the passage of proteins over this filtration barrier, there are three selective criteria that are of importance namely, a protein's molecular size, its total molecular charge and its concentration in the blood. Alternatively the primary factors influencing the passage of proteins across the glomerular filtration barrier can be expressed as the size-selective and charge selective properties of the glomerulus as well as the haemodynamic forces operating across the glomerular capillary walls (Lulich and Osborne, 1990).

2.1.1.1 Molecule size

Although the pores in the capillary endothelium are generously large (70-100 nm) it is the pore size in the last layer of the glomerular filtration barrier that actually determines the passage of molecules. Brenner et al (1978) used dextrans with varying molecular sizes to investigate the relationship between molecule size and filtration. They reported that molecules with effective radii of less than 1.4 nm have free passage so that the concentration of such substances in the Bowman's capsule is equal to that in plasma. Furthermore, it was also shown that molecules with a relative radius greater than 3.6 nm, the approximate size of albumin, have decreased clearance and only low amounts are found in the ultrafiltrate. Substances whose radius exceeds 4.2 nm have zero clearance. More recently it has been suggested that the average radius of the pores in the slit membrane is around 2 nm, so that when based on size alone, the passage of albumin is largely restricted. The glomerular barrier may also have a small number of pores with a radius of 4.5-5 nm and about 1% of the pores may in fact be as large as 8-10 nm in radius (Haraldsson and Sorensson, 2004).

2.1.1.2 Molecule charge

Passage across the glomerular filtration barrier is not determined solely by the size of the molecules and pores, but is also dependent on the combination of size with the charge characteristics of both the transportable molecules and the barrier structures (Haraldsson and Sorensson, 2004). Acidic glycoproteins or sialoproteins, expressed in all three layers of the glomerular filtration barrier provide the barrier with a net negative charge. As the majority of plasma proteins at physiological pH are polyanionic structures, the barrier's net negative charge works as an electrostatic hindrance to their passage (Brenner et al., 1978; Barber et al., 1996; Raila et al., 2005). In addition to this, on the endothelial cell surface is a layer of proteoglycans, glycosaminoglycans (GAGs) and plasma proteins, which are produced by the endothelial cells themselves and is known as the glycocalix. The interaction between the proteoglycans, GAGs and the proteins gives this approximately 300 nm thick layer its gel-like consistency and its size- and charge-selective properties (Haraldsson and Sorensson, 2004). Due to these features and based on studies involving the sieving characteristics of neutral and polyanionic dextranes, it has been shown that neutral dextranes pass the glomerular filtration barrier more easily than same sized negatively charged dextranes (Waller et al., 1989). Independent of size, polyanionic substances are generally more restricted in their filtration and the combination of polyanionic charge and large molecular size decreases clearance to a great extent (Brenner et al., 1978).

2.1.1.3 Haemodynamic force

The third determining factor for passing the glomerular filtration barrier is the concentration of a particular protein in plasma. If the concentration is high the hindering factors of size and charge can be overcome to some extent, as is the case for albumin. Although albumin is polyanionic, has a molecular weight of 69 kDa and a radius of 3.6 nm (Brenner et al., 1978) with an effective radius of 7.5 nm (Peters, 1985; Gekle, 1998; Gekle, 2005) which should render it unsuitable for passage over the glomerular filtration barrier, it is still found in normal canine urine in concentrations of up to 40-60% of the total urinary proteins (Barsanti and Finco, 1979; Lulich and Osborne, 1990). Albumin represents 60% of plasma proteins and has a plasma concentration for humans of approximately 45 g/l (Peters, 1985). This results in a high haemodynamic force and enables 0.05-0.1% of plasma albumin to reach the glomerular ultrafiltrate. Up to 45 mg/l of albumin can be present in the ultrafiltrate of humans (Gekle, 1998). The fact that under physiological conditions this amount of albumin is not present in end urine is due to the reabsorption that takes place in the following tubule sections of the nephrons.

2.1.2 Tubular reabsorption of glomerular filtered proteins

The lumen of the Bowman capsule extends into the renal tubules. This is a system comprised of a proximal tubule with a convoluted and a straight section, the Henle`s loop with its descending and ascending limbs and a distal tubule with a straight then convoluted section, which finally enters the collecting tubule. The collecting tubules run parallel to the Henle`s loop and service more than one nephron before leading into the renal pelvis (Liebich, 1999).

The reabsorption process begins in the proximal convoluted tubules. For this purpose, the epitheliums of the tubules have a structure similar to other resorptive epithelial tissues (Christensen et al., 1998; Raila et al., 2005). The apical cell surfaces are covered with microvilli, in the form of a “brush border membrane” which hugely enlarges the surface area. These cells are also rich in surface receptors and vesicles such as lysosomes which are necessary for intra-epithelial substance transport. 75% of the ultrafiltrate is isotonically reabsorbed in the proximal tubule along with substances such as sodium, potassium, calcium, phosphorus, glucose and amino acids (Finco and Duncan, 1976). The reabsorption of these substances occurs passively according to concentration gradients, as is the case for electrolytes or actively under expenditure of energy, as is the case for glucose and amino acids. The distal tubules further complete the refining of urine to a highly concentrated and hypertonic fluid as well as balancing electrolyte levels and even secreting certain proteins (Liebich, 1999).

2.1.2.1 Receptor-mediated endocytosis in the proximal tubules

Although low molecular weight proteins of up to 40 kDa in size are freely present in the glomerular ultrafiltrate, under physiological conditions such proteins are virtually no longer found in fluid after it has passed through the proximal tubule (D`amico, 2003; Christensen and Gburek, 2004). These proteins are reabsorbed in the following three ways: Exopeptidases which are expressed on the apical cell membranes in the proximal tubules are responsible for cleaving small proteins, up to 10 kDa in size, to amino acids or di-peptides which are then reabsorbed with the help of sodium dependent transport systems (Gekle, 1998; Raila et al., 2005). Endocytosis is responsible for a greater portion of protein reabsorption. This occurs either through “fluid-phase endocytosis” whereby tubular fluid containing dissolved molecules is caught in membrane invaginations which are transported as endocytic vesicles through endosomal then lysosomal compartments before further catabolism. The concentration of substances in these endocytic vesicles is exactly the same as that in the tubular fluid and this method represents therefore, a rather general method of

reabsorption, which is slow and quantitatively negligible (Gekle, 1998; Gekle, 2005). A much more specific method however, is “receptor-mediated endocytosis” whereby substances are concentrated at the cell membrane through binding on specific receptors. The receptors involved in this quantitatively highly effective method are megalin and cubilin (Gekle, 1998; Christensen and Birn, 2001; Verroust and Christensen, 2002; Gekle, 2005) and immunological and histological studies have shown both receptors to be present on the apical membrane of the proximal tubules of healthy dogs (Raila et al., 2003).

2.1.2.2 Megalin

Megalin is a 600 kDa multi-ligand transmembrane endocytic receptor belonging to the family of low density lipoprotein (LDL) receptors and appears in the kidneys mostly on the apical surface of the proximal tubule epithelium (Christensen and Birn, 2001; Raila et al., 2003; Raila et al., 2005). It is also expressed in other non-renal absorptive epithelial cells with contact to transcellular fluids, such as the choroid plexus, thyroid cells and the inner ear labyrinth (Christensen and Birn, 2001; Verroust and Christensen, 2002; Christensen and Gburek, 2004). Megalin is known as a “scavenger receptor” due to its high number and wide variety of acceptable ligands, including albumin and the vitamin-binding proteins, retinol-binding protein (RBP), vitamin D-binding protein (DBP) and transcobalamin II as well as other carrier proteins and hormones as shown in Table 1. Megalin is the most important receptor for endocytosis and reabsorption of RBP (Nykjaer et al., 2001). This has been shown in experiments with megalin-deficient mice who have higher levels of vitamin-binding proteins in their urine as well as micronutrient loss (Christensen et al., 1999; Raila et al., 2005). Megalin also binds very strongly to calcium and the binding of many other ligands is in most cases calcium dependent (Verroust and Christensen, 2002). The binding affinity of ligands is dependent on the pH level of the proximal tubular fluid which must be between 7.4 and 6.7 for binding to take place as well as being greatly facilitated by cationic binding sites on the ligands themselves (Christensen and Birn, 2001; Gekle, 2005). Once receptor-ligand-complexes have been internalized these “early endosomes” must be acidified to initiate the ligand-receptor dissociation. This is achieved through vacuole-type-H^+-ATPases and counter-ion conductance through chloride channels. The proteins can then be transferred to lysosomes for degradation and the receptors return to the apical membrane surface in “recycling endosomes” (Gekle, 2005). In the also acidic environment of the lysosomes, the proteins are cleaved and the resulting amino acids and small peptides transverse the basolateral membrane to re-enter the blood stream for further use (Gekle, 1998; Gburek et al., 2003; Gekle, 2005).

2.1.2.3 Cubilin

Cubilin is a 460 kDa peripheral membrane receptor which is identical to the intrinsic factor-vitamin B_{12} receptor in the small intestine. As cubilin has no cytoplasmic domain it is dependent on receptor-receptor interaction and co-internalization with megalin to mediate the endocytosis of cubilin and its ligands (Christensen and Gburek, 2004). Ligands of cubilin include albumin, DBP and transferrin however neither RBP, nor β_2-microglobulin are amongst its ligands (Christensen and Birn, 2001). Cubilin is essential in preventing proteinuria due to its role in albumin tubular reabsorption. This has been shown in studies on dogs with genetic cubilin defects who demonstrate albuminuria as well as vitamin B_{12} malabsorption and abnormal vitamin D metabolism (Christensen and Birn, 2001; Nykjaer et al., 2001; Verroust, 2002). The iron carrier proteins, transferrin, myoglobin and haemoglobin are ligands for both receptors although cubilin has a lower affinity for haemoglobin (Gburek et al., 2002). Investigations using immuno-histochemistry and fluorescence-labelled haemoglobin have shown that megalin-mediated reabsorption of haemoglobin occurs in physiologic conditions and that the ability of cubilin to reabsorb haemoglobin gains importance during haemolytic conditions during which the haemoglobin concentration in the ultrafiltrate is increased and high capacity uptake is necessary (Gburek et al., 2002).

Table 1: Ligands for megalin and cubilin (modified from Verroust and Christensen 2002)

Megalin and Cubilin	**Megalin specific**	**Cubilin specific**
Albumin	RBP	Transferrin
Haemoglobin	Transcobalamin-vitamin B_{12}	Apolipoproteins A-I
DBP	β2-Microglobulin	HDL
Ig light chains	Transthyretin	
Apolipoprotein B	PTH	
	Ca^{2+}	

DBP= Vitamin D-binding protein; RBP=Retinol-binding protein; PTH= Parathyroid hormone;
HDL= High density lipoprotein

This system of receptor-mediated reabsorption of proteins does have limitations. For instance, the binding affinity of the albumin binding sites in the proximal tubules is in a similar range to the physiological tubular albumin concentration, of which 99% is reabsorbed from the ultrafiltrate (Gekle, 1998). This means however that the system can become saturated. If the concentration of ligands is higher than the binding affinity, excess proteins can not be reabsorbed and will therefore be present in higher amounts in the end urine (Christensen and Gburek, 2004).

2.1.3 Tubular secretion of proteins

Independent of the glomerular filtration and proximal tubular reabsorption of proteins, some proteins are produced and secreted by the epithelial cells in the distal tubule, ending up thus in the urine. The most quantitative of these proteins present in the physiological proteinuria in dogs, is the Tamm-Horsfall protein. This 100 kDa glycoprotein is synthesized by the epithelial cells in the distal tubules and is believed to have antiviral qualities (Waller et al., 1989; Raila et al., 2005). Urokinase, a fibrinolytic enzyme is also secreted by tubular epithelial cells as is the 300 kDa Immunoglobulin A (Ig-A) (Waller et al., 1989; Lulich and Osborne, 1990). A certain amount of proteinic substances also end up in urine in the course of its passage through the lower urogenital tract, particularly in the case of infection in this region although certain prostate proteins are also present under physiological conditions in the urine of male dogs (Teinfalt et al., 2000; Tsuchiya et al., 2005).

2.2 Physiological proteinuria

Whilst bearing in mind the physiology of the kidneys and their anatomical characteristics as mentioned above, it is still normal for urine to contain small quantities of proteins. Physiological proteinuria in humans is in the range of up to 150 mg per day (Waller et al., 1989) containing 30-40% albumin, 5-10% IgG, 5 % globulin light chains, 3 % IgA and THP making up the remainder (Maachi et al., 2004). Various studies have also been undertaken to determine the physiological level of protein in canine urine. Barsanti and Finco (1979) tested 193 urine samples from dogs without symptoms of urinary tract disease and concluded that, regardless of the specific gravity, canine urine should contain less than 65 mg/dl protein. He also noted that samples from male dogs, obtained after voluntary micturition were significantly higher in protein than those from females, whereby no difference was observed in cystocentesis or catheter samples. Another author however, who investigated differences in proteinuria between desexed and non-desexed male dogs and female dogs, discovered no difference in their urinary protein levels (Teinfalt et al., 2000).

2.3 Pathological proteinuria

When considering the mechanisms controlling renal protein passage, pathological proteinuria, commonly referred to simply as proteinuria, occurs when the passage of proteins over the glomerular filtration barrier is increased and/or the reabsorption processes in the proximal tubules are impaired (D`amico, 2003). In addition to this, there are also pre- and post-renal causes of proteinuria. An increased amount of protein in urine on its own however,

is a rather non-specific marker for the state of renal function. Analysis of the pattern and qualities of the proteins contained can provide information on which area of the nephron is responsible for the protein loss (Maachi et al., 2004). Also of importance, particularly when the proteinuria is only marginal, is the question of whether it is transient or persistent (Elliott, 2006). Based on the protein pattern, proteinuria can be classified according to the anatomical site at which the excess protein enters the urinary tract (Barber et al., 1996). With this knowledge, it is possible to partly differentiate between the 5 types of pathological proteinuria, namely: (1) overload or pre-renal, (2) glomerular, (3) tubular, (4) mixed renal and (5) proteinuria due to postrenal causes.

2.3.1 Pre-renal proteinuria

Pre-renal or overload proteinuria occurs when excessive amounts of low molecular proteins (LMW) are circulating in the blood. These proteins are filtered automatically through the glomeruli and are consequently present in high amounts in the ultrafiltrate. The reabsorptive capacity of the proximal tubule can herewith be exceeded and higher than normal amounts of such proteins reach the end urine (Waller et al., 1989). This situation occurs for example, during high levels of intravascular haemolysis which leads to an increase in free haemoglobin (15 kDa) in plasma and consequent filtration by the glomeruli (DiBartola, 1980; Christensen and Gburek, 2004). Similarly, following trauma, muscle damage or rhabdomyolysis the resultant excessive amounts of circulating myoglobin (17 kDa) are freely filtered. Alternatively, overload proteinuria occurs in the case of multiple myeloma, a neoplastic B-cell disorder characterized by high production of immunoglobulin light chains, known as Bence Jones proteins. These LMW proteins (22 kDa monomer, 44 kDa dimer) (Lulich and Osborne, 1990) are freely filtered, leading to proteinuria and the subsequent use of these proteins as a marker for this illness (DiBartola, 1980; Christensen and Gburek, 2004; Maachi et al., 2004).

Functional transient proteinuria is a type of pre-glomerular proteinuria that occurs after strenuous exercise, during fever, seizures or in a state of hypothermia and is associated with high albuminuria in humans (Vaden, 2004). To date, there exists only sparse information as to whether this is the case for dogs, however one recent study involving the measurement of proteinuria before and after exercise indicates that at most, 15% of dogs can be expected to develop microalbuminuria after exercise. Furthermore, dogs with pre-existing glomerular damage were the most likely candidates for exercise induced microalbuminuria (Gary et al., 2004; Vaden, 2004).

2.3.2 Glomerular proteinuria

Glomerular proteinuria occurs when the selective permeability of the glomerular filtration barrier is altered in some way as to allow the filtration of molecules whose size or combined size and charge would normally not permit this. The level of protein in the urine is usually quite high (Waller et al., 1989) and the proteinuria is characterized by the presence of middle (MMW) to high (HMW) molecular weight proteins, larger than 60 kDa (DiBartola, 1980). “Selective” glomerular proteinuria occurs when the glomerular damage is only moderate due to loss of the charge selectivity of the GBM and contains MMW proteins in the range of 60 kDa to 80 kDa (e.g. transferrin). As the disease progresses the proteinuria becomes “non-selective” and contains HMW proteins larger than 78 kDa such as IgG (Waller et al., 1989; Lulich and Osborne, 1990; Yalcin and Cetin, 2004).

Factors that are believed to alter the permeability of the glomerulus are: an increase in pore size, an increase in the number of pores or a change in the charge characteristics, increases in intraglomerular and hydrostatic pressure, the malfunction of podocyte-associated molecules such as nephrin or podocin and the infiltration with inflammatory cells (Waller et al., 1989; Barber et al., 1996; Ruotsalainen et al., 1999; Perico et al., 2005). The mechanisms behind such alterations often begin as a reaction to non-renal disease processes. Underlying infections, inflammatory processes, metabolic conditions, the presence of neoplasia as well as the toxic effect of some medications can secondarily cause alterations in glomerular permselectivity. Known causes of glomerular malfunction in this way are renal amyloidosis and glomerulonephritis (Lees, 2004).

In renal amyloidosis, extra cellular serum amyloid A is deposited in the glomeruli as a reaction to inflammation, neoplasia or chronic infection. Chinese Shar Pei dogs and Abyssinian cats are known to suffer from renal amyloidosis (Barber et al., 1996). Glomerulonephritis is caused by the deposition of antibody-antigen complexes on the GBM (Waller et al., 1989). Brenner et al. (1978) studied the effect of experimentally induced nephrotoxic serum nephritis and also the application of puromycin aminonucleoside on rat kidneys and found that alterations in the intrinsic charge properties of the glomerular capillary wall caused changes in the filtering capabilities of the glomerulus and led herewith to substantially greater clearances of polyanionic substances regardless of their molecular size. Mutual electrostatic repulsion ensures that the foot processes are normally thin and finger-like. When the repulsing charges are removed as is the case when antibody-antigen complexes interact with glomerular proteoglycans, the epithelial foot processes become fused together and the pores in the slit membrane become enlarged. In this context, Brenner et al. (1978) also mentions

the effect of decreased glomerulum cellular sialoprotein content. This also led to a loss in the negative charges on the plasma membrane surface, facilitating once again a fusion of the normally self-repelling foot processes.

Glomerular damage can also be caused by the deposition of circulating antigen-antibody complexes in the glomerulum, the deposition of antigens possessing a biochemical affinity for molecules in the glomerular capillary walls and the formation of immune complexes within the glomerular capillary (Grauer, 2005). Electrical charge interaction between antigens and molecules in the glomerulum can lead to stimulated production of pro-inflammatory cytokines, vasoactive substances, growth factors and proteases within the kidneys, exacerbating glomerular damage (Perico et al., 2005). In turn, the renin-angiotensin-aldosterone system (RAAS) is activated and causes vasoconstriction of the efferent glomerular capillary leading to intra-glomerular hypertension. This results in an increase in hydrostatic pressure, further driving proteins over the glomerular filtration barrier. Platelets, activated through the endothelial damage, release more vaso-constrictive and inflammatory substances, compounding the problem. Additionally, a chronic accumulation of proteins, particularly in the glomerular tuft can exacerbate the glomerular damage as their presence leads in the long term to stimulation of growth factors, proliferation of the endothelium, thickening of the GBM and fibrosis (Gekle et al., 2003; Gekle, 2005; Perico et al., 2005).

2.3.3 Tubular proteinuria

Tubular proteinuria occurs when filtration is normal but reabsorption is incomplete due to impairment of the resorptive mechanisms in the proximal tubular epithelium. Tubular proteinuria is characterized by the presence of LMW proteins (<60 kDa) such as RBP (21 kDa), α1-microglobulin (31 kDa) and β2-microglobulin (12 kDa), which under normal circumstances freely pass the glomerular filtration barrier but should be reabsorbed in the proximal tubule. Tubular proteinuria is usually characterized by lower levels of protein than proteinuria of a glomerular nature (Bernard et al., 1987; Waller et al., 1989; Lulich and Osborne, 1990; Yalcin and Cetin, 2004).

A number of genetic anomalies in humans are known to impair tubular reabsorption. These include Dent's disease whereby the chloride channels in the endosomes are defect so that endosomal acidification fails (Gekle, 2005) and Imerslund-Gräsbeck syndrome where the cubilin gene is mutated so that its expression and distribution, both in the kidneys and the intestine is inadequate (Christensen and Gburek, 2004). Both Dent's disease and the Imerslund-Gräsbeck syndrome are characterized by excessive excretion of LMW proteins (Christensen and Gburek, 2004). There is also evidence that a canine form of Imerslund-

Gräsbeck syndrome exists with selective vitamin B12 malabsorption and proteinuria (Verroust, 2002; He et al., 2003).

Tubular proteinuria can also occur during conditions such as acute tubular necrosis, renal infarction and bacteria-associated interstitial nephritis (Waller et al., 1989). Several toxins, such as Pertussis toxin and ochratoxin are also known to inhibit endocytosis (Gekle et al., 1998). Furthermore, certain drugs, such as non-steroidal anti-inflammatory drugs (NSAIDS), aminoglycosides and cyclosporins as well as the chemical toxins ethylene glycol, cadmium and lead can have a toxic effect on the tubular epithelium cells (Nelson and Couto, 2003).

2.3.4 Mixed or tubulo-glomerular proteinuria

Mixed proteinuria occurs when both glomerular filtration and tubular reabsorption are disturbed. If abnormally high amounts of proteins pass the glomerular filtration barrier, the competitive affiliation for megalin and cubilin can reach saturation point (Thelle et al., 2006). Tubular reabsorption can no longer keep pace and increasing amounts of LMW, MMW and HMW proteins become present in the end urine. Mixed proteinuria can also occur as the consequence of chronic glomerular proteinuria leading to tubular damage. The continued presence of large amounts of protein in the ultrafiltrate necessitates high reabsorption and excessive lysosomal processing. Lysosomal rupture and consequent enzymatic damage to epithelial cells further inhibits protein uptake (Gekle et al., 1998; Gekle et al., 2003). Certain HMW plasma proteins which should normally not reach the renal tubules in such high amounts can also instrument damaging reactions within the tubular epithelial cells. For instance, when high levels of transferrin are reabsorbed, the increased epithelial cell iron content contributes to the formation of damaging oxygen radicals. Prolonged exposure of the apical surface of tubular cells to high levels of plasma proteins results in upgraded expression of growth factors which cause tubulo-interstitial inflammation, fibrosis and the further deterioration of both glomerula and tubules (Berridge, 2004; Grauer, 2005). This is reported in Bernese mountain dogs with a form of juvenile renal disease. Severe membranous glomerulonephritis leads to tubulopathy and interstitial fibrosis, characterized by the excretion of marker proteins for both glomerular damage and proximal tubular dysfunction (Raila et al., 2007).

2.3.5 Postrenal proteinuria

In this case, proteins resulting from inflammation, lesions, neoplasia, ischemia or trauma in the lower urogenital tract are found in urine (Waller et al., 1989; Lulich and Osborne, 1990). This form of proteinuria can be diagnosed with relative ease due to the accompanying clinical

symptoms, and examination of urine sediment, which usually contains high amounts of erythrocytes and/or leukocytes which are absent in urine from glomerulonephropathies or the presence of very large proteins in the urine without symptoms of renal failure (Lulich and Osborne, 1990). One study using sodium dodecyl sulphate-agarose gel electrophoresis (SDS-AGE) to examine urine from critically ill human patients with acute renal failure indicates that the 725 kDa α2-macroglobulin is a marker for postrenal proteinuria (Gai et al., 2004). Several proteins of post renal origin have been detected in the urine of male dogs. A 30 kDa prostate specific protein (PSP) is the main protein in canine seminal plasma (Teinfalt et al., 2000) and a 15 kDa prostatic tissue kallikrein precursor, produced in the prostate gland, is reportedly present as a distinct double band at approximately 15 kDa in SDS-PAGE of urine from normal male dogs (Tsuchiya et al., 2005). Immuno-histochemical investigation has shown that whilst PSP concentration in urine of healthy dogs is varied, its secretion is hormonally dependent, as the protein was not identified in urine from bitches or castrates. The amount of PSP in urine is reportedly indicative of various forms of prostate illness (Teinfalt et al., 2000).

2.4 Systemic inflammatory response syndrome (SIRS)

2.4.1 SIRS in humans

2.4.1.1 Definition and epidemiology

SIRS was defined in a 1992 consensus conference of the American College of Chest Physicians and the Society of Critical Care Medicine (ACCP/SCCM) as the systemic inflammatory response to a variety of severe clinical insults of both infectious and non-infectious natures. A scheme of this definition is depicted in figure 1. Patients are identified as having SIRS when they exhibit two or more of the clinical signs listed in table 2 (Bone et al., 1992). In the consensus definitions the term sepsis was reserved for SIRS resulting from a confirmed infectious process. Additionally, the complications associated with SIRS and the progression of the disease complex to severe sepsis, septic shock and multiple organ dysfunction were defined (table 3). More recently some authors have suggested that the actual incidence of primary sepsis or number of SIRS patients developing secondary sepsis is underestimated due to the practice of applying empirical antimicrobial therapy to many SIRS patients (Brun-Buisson, 2000). Sepsis has therefore more recently been defined as both a confirmed as well as clinically suspected infection accompanied by two or more SIRS criteria (German Sepsis Society, 2006; Levy et al., 2003).

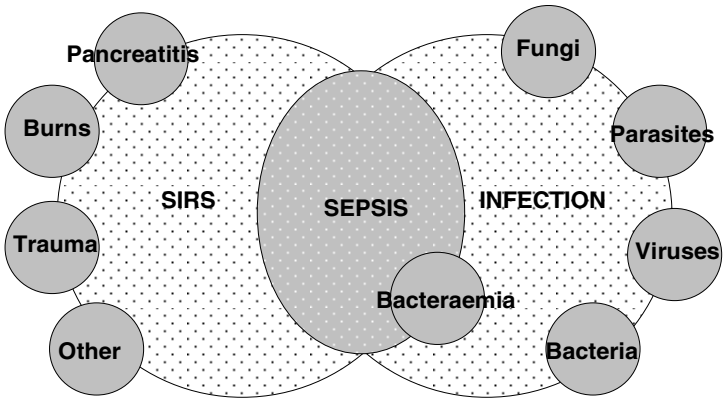

Figure 1: The development of SIRS from infectious and non-infectious insults and the interrelationship with sepsis, (modified from Bone et al, ACCP/SCCM consensus conference 1992).

Table 2: Symptoms used in the identification of SIRS in humans, (modified from Bone et al 1992).

Clinical parameters	**Reference values**
Temperature (℃)	> 38 or < 36
Heart rate (beats/min)	> 90
Respiration rate (breaths/min)	> 20 or PaCo2 < 32 mmHg
WBC count (cells/µl)	> 12 000 or < 4000 or 10 % immature forms

Table 3: Definition of the SIRS complex, (modified from Bone et al 1992 and Nyström 1998).

Term	Definition
SIRS	The systemic inflammatory response to a variety of severe clinical insults, as manifested through the presence of 2 or more of the SIRS criteria
Sepsis	SIRS plus evidence of, or strong suspicion of infection
Severe sepsis	Sepsis plus evidence of organ dysfunction
Septic shock	Sepsis plus hypotension despite adequate fluid replacement
MODS	Diminished function of more than 2 organs/organ systems (cardiovascular, respiratory, renal, hepatic, haematological, neurological, gastro intestinal tract) so that homeostasis cannot be maintained without intervention.

Abbreviations: SIRS, systemic inflammatory response syndrome; MODS, multiple organ dysfunction syndrome

The incidence of SIRS in intensive care unit (ICU) patients throughout Europe was recently reported to be as high as 93% (Sprung et al., 2006). A 2001, joint European and American intensive care consensus conference, which positively reviewed the 1992 definitions, underscored further the still existing challenge posed by SIRS and sepsis. The authors report that severe sepsis is the most common cause of death in non-coronary intensive care units throughout Europe and America (Levy et al., 2003). In human ICUs the use of standard scoring systems has been incorporated to evaluate a critical patient's condition and to allow the comparison between patients and study populations. Such systems are the APACHE II (acute physiology and chronic health evaluation) and SOFA (sequential organ failure assessment), (Knaus et al., 1985; Vincent et al., 1996).

2.4.1.2 Aetiology of the SIRS complex

Immediately following tissue injury, the acute phase cytokines interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α) and to a lesser extent, IL-6 and IL-8 appear rapidly in circulation (Barriere and Lowry, 1995; Nyström, 1998). Cytokines are LMW proteins which act on surface receptors of various target cells to initiate and mediate further immune action (Desborough, 2000). The rapid, non-pathogen-specific "cell-mediated innate immunity" involves neutrophilic granulocytes, macrophages, monocytes, natural killer cells and the complement system (Smith et al., 2006). Neutrophilic granulocytes and macrophages perform phagocytosis, whilst macrophages are responsible for antigen presentation and herewith, the priming of T-cells. Macrophages and natural killer cells also stimulate the further release of IL-6, TNF-α, prostaglandin E-2 (PGE-2), interferon gamma (IFN-γ) and IL-12 (Bone et al., 1992; Brun-Buisson, 2000; Smith et al., 2006). In this way the long-term pathogen-specific "adaptive immune system" is activated, involving T-lymphocytes, the membrane attack complex (MAC) and anaphylotoxins (Smith et al., 2006).

The pro-inflammatory response is believed to help aid the preservation of body functions during acute and serious insults (Brady and Otto, 2001). Once an insult has been effectively controlled, the activation of anti-inflammatory mechanisms is necessary in order to attenuate the response and allow a return to normal (Nyström, 1998). Under normal circumstances the pro-inflammatory response should be kept in check by compensatory anti-inflammatory mechanisms (Smith et al., 2006). Such mechanisms include down-regulating the production of pro-inflammatory cytokines, de-sensitising receptor cells for pro-inflammatory cytokines and up-regulating production of anti-inflammatory cytokines. For instance, IL-10, the prototype anti-inflammatory cytokine is responsible for decreasing macrophage activity and suppressing macrophage ability to present antigens (Cohen, 2002). The two types of T-helper cells (Th-1 and Th-2) produce antagonistic cytokines and illustrate therefore the

close relationship between pro- and anti-inflammatory mediators (Smith et al., 2006). It is believed that the severity of an inflammatory reaction develops in accordance with the severity of the patient's injuries. In such cases of extreme insult, the primed immune system can overreact with a massive production of pro-inflammatory cytokines (Gopal et al., 2006; Smith et al., 2006). The feedback systems between pro- and anti-inflammation should restore balance, but in this situation the anti-inflammatory response may be insufficient or alternatively over compensatory and an imbalance can arise. Figure 2 depicts the relationships between the pro- and anti- inflammatory responses at the local and systemic levels and their influence on the development of SIRS.

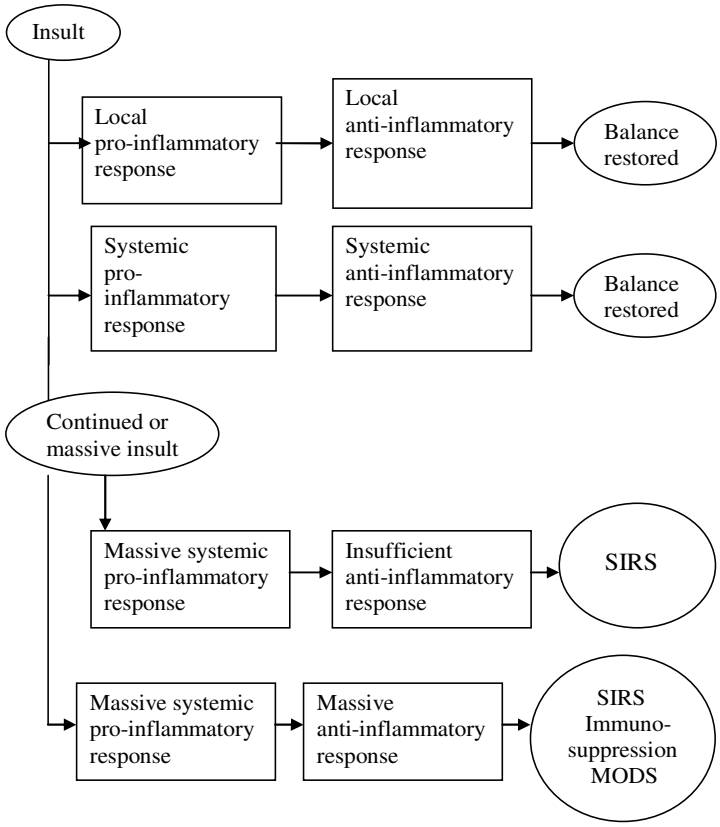

Figure 2: The four stages of inflammatory response with local and systemic reactions progressing to the development of SIRS and MODS, (modified from Bone, 1996)

As part of their normal function TNF-α, IL-1 and neutrophilic granulocytes cause activation of the endothelium cells which in turn leads to increases in the permeability of blood vessels (Abid et al., 2001). The membrane attack complex (MAC) and anaphylotoxins also cause endothelial cell activation (Smith et al., 2006). Activated endothelium cells produce nitric oxide (NO) which causes vasodilatation (Groeneveld et al., 1997). Locally, increased permeability and vasodilatation cause disturbances in microcirculation and lead to oedema,

erythema as well as pain. On a systemic level however, vasodilatation and increased capillary permeability can lead to large-scale vascular leakage. Consequent hypovolaemia, hypotension, hypoperfusion and tissue hypoxia are believed to be the instrumental factors in the eventuation of shock and multiple organ dysfunction (Bone, 1996; Brady and Otto, 2001; Cohen, 2002; Smith et al., 2006). Another exacerbating factor behind organ dysfunction is SIRS induced hypercoagulation. Endothelial cells release thrombogenic tissue factors which activate coagulation cascades, whilst anticoagulative pathways are down-regulated and the production of endogenous anticoagulants can be reduced (Cohen, 2002; de Laforcade et al., 2003). The consequent development of microthrombi in blood vessels may hinder tissue perfusion, leading once again to ischaemic organ damage. “Toxic” damage to organs can also occur during cytokine-mediated apoptosis, the release of lysosomal enzymes by the infiltrating neutrophilic granulocytes and the accumulation of reactive oxygen products in hypoxic cells (Spapen et al., 2005). Furthermore following reperfusion of ischaemic areas, oxidative stress may occur due to the depletion of endogenous antioxidants (Johnson, 2004). Hypoxia induced endothelial cell damage is also believed to play a large role in the secondary development of sepsis during SIRS as it aids the translocation of intestinal bacteria into mesenteric circulation (Sherwood and Toliver-Kinsky, 2004).

2.4.1.3 The effect of SIRS on renal function

The increased passage of proteins over the glomerular filtration barrier and herewith the occurrence of microalbuminuria (MA) or overt proteinuria is reportedly a result of the SIRS induced vasodilatation and increased capillary permeability in the kidneys. To this extent, some authors have proposed that the level of MA is an indicator of the severity of generalized increased vascular permeability during SIRS (Gosling et al., 1994; De Gaudio et al., 1999). MA has therefore been investigated as a marker for SIRS and has been proposed to reflect not only illness severity, but also the potential for the occurrence of complications and for the prediction of mortality (MacKinnon et al., 2000; Abid et al., 2001). The search for markers for SIRS arises not only out of the need for rapid identification of patients most at risk of developing complications, but also prospectively for reliable SIRS identification using biochemical and/or immunological parameters rather than depending on clinical criteria (Levy et al., 2003). Although various inflammatory mediators have been suggested as markers, some authors report that the cytokine response in sepsis patients is very inhomogeneous rendering cytokines therefore less suitable as markers (Damas et al., 1997; Gosling et al., 2003). The method for measuring MA is quick, simple, inexpensive and non-invasive, which has contributed to its proposed usage as a marker.

As previously mentioned, one of the earliest features of inflammation is the increased permeability of capillaries for plasma proteins, which leads to their increased excretion over the glomerular filtration barrier (Fleck et al., 1985; Gosling et al., 2003). Under physiological conditions the tubular albumin concentration is already close to that which can be readily reabsorbed, and given that 99% of physiologically filtered albumin in the ultrafiltrate is actually reabsorbed (Gekle, 1998), the range between normal tubular albumin concentration and saturation point is very narrow. A small increase in permeability can therefore have the net result of a large increase in albumin excretion (Abid et al., 2001). MA is believed to occur within minutes of the start of inflammatory response and provided there is no worsening of the SIRS state, proteinuria should return to normal within 6 -12 hours according to one study (Gosling et al., 2003) or within 48 hours according to others (Abid et al., 2001). Most of these recent investigations were conducted using catheter urine, immunoassay for detection of albumin in urine and the examination of a urine albumin to urine creatinine ratio (UAC) to correct for variation in the urine flow. In human medicine the use of standard scoring systems to evaluate a patient's conditions and allow comparison of patients and study populations has also been incorporated into the evaluation of markers for SIRS. Such systems are the APACHE II and SOFA (Knaus et al., 1985; De Gaudio et al., 1999; Abid et al., 2001; Gosling et al., 2003; Thorevska et al., 2003; Gopal et al., 2006).

Recent human studies on the occurrence of MA in critically ill patients and its use as a marker for illness severity and prognosis concur that MA is a regular finding in ICU patients. However, the actual predictive value of MA and its correlation with illness severity are a subject of dispute. In one study (MacKinnon et al., 2000), urinary albumin levels for both survivors and non survivors were increased at admission; however the UAC from urine obtained 6 hours later was significantly higher for non-survivors. Furthermore, the degree of MA for non-survivors correlated with the severity of APACHE and SOFA scores so that these authors concluded that the level of MA was significantly associated with outcome. Another study (Gosling et al., 2003) compared the predictive values of a UAC obtained within 15 minutes of admission with the illness severity scores obtained after 24 hours. For trauma, burns and surgical patients, UAC as well as illness severity scores were significantly higher in non-survivors than survivors. The level of MA immediately on admission was shown to be as good a predictor of mortality as the scoring systems used after 24 hours. The authors report that for these patients, the UAC had a 100% predictive value for survival when the inflammatory response was modest and transient inflammatory whilst for patients with an exaggerated response, it had a 25% predictive value for death. However, for purely medical patients, neither UAC nor illness severity scores showed a difference between survivors and non-survivors, so that the UAC did not have any predictive value for mortality. Along similar

lines, Thorevska et al (2003) examined urine from non-surgical ICU patients at admission and after 48 hours. 69% of the examined patients had microalbuminuria or overt proteinuria and the level of MA was shown to reflect the illness severity scores. The authors also proposed that the level of UAC and the mortality rate may underlie the influences of race, age, the presence of diabetes mellitus, shock, sepsis or renal insufficiency. In this study though, a threshold value of 100 mg/g (3 times the upper limit for normal MA) was nominated and the authors state that patients with a UAC above this level had a higher mortality rate.

In a further study involving non-surgical ICU patients with both SIRS and sepsis (Abid et al., 2001) MA was investigated for its predictive value in the development of acute respiratory failure and multiple organ failure. The authors observed the trend in MA values over the first 48 hours and based on this, the patients were divided into two groups; group 1 with increasing MA and group 2 with decreasing or stable MA during this period of time. The hospital mortality rate for group 1 was 43% and for group 2, 15%. Patients with increasing MA had generally higher APACHE and SOFA scores and a clearly much higher occurrence of (multiple) organ failure compared to patients who's MA decreased. Although the initial MA level in group 2 patients was sometimes higher than in group 1, the decreasing course of their MA over the first 48 hours was deemed to be more important for their favourable outcome rather than the severity of the initial MA itself.

In contrast to all the previously mentioned studies DeGaudio et al (1999) reported a far more inconclusive use for MA as a marker for mortality. These authors investigated albuminuria in trauma patients in an ICU to determine whether a correlation exists between an increase in glomerular permeability, the magnitude of trauma and the mortality rate. Urine was collected at admission and up to 24 hours thereafter and an injury severity score (ISS) was calculated at admission as well as the APACHE and SAPS scores after 24 hours. They reported that although the UAC measured at 24 hours after trauma was above normal, there was no significant difference in UAC between survivors and non-survivors. Although the MA severity was significantly correlated with the severity of the insult (ISS score) the level of MA did not have any predictive value as to outcome and mortality.

2.4.2 Occurrence of SIRS in dogs

To date very little research has been undertaken dealing directly with the incidence, diagnosis, ensuing complications such as organ dysfunction and outcomes of SIRS in companion animals. The understanding of SIRS in a veterinary context therefore is still based largely on information from human medical literature. This is acknowledged by Brady and Otto (2001) who report that SIRS in dogs is known to occur after pancreatitis,

heatstroke, snake bites, neoplasia, trauma, surgery and burns. Based on their studies and a study of 350 dogs by Hauptman et al. (1997), SIRS in dogs can be diagnosed by the presence of two or more of the symptoms listed in Table 4.

Table 4: Symptoms used in the identification of SIRS in dogs, (modified from Brady and Otto, 2001 and Hauptman et al 1997).

Clinical parameters	**Reference values**
Temperature (℃)	small dogs > 39.4 or < 38
	large dogs > 39.3 or < 37.8
Heart rate (beats/min)	> 120
Respiration rate (breaths/min)	> 20
WBC count (cells/µl)	> 16 000 or < 6000
	or > 3% juvenile forms

Recognized localisations or focal points of primary sepsis in dogs include peritonitis, pyometra, pancreatitis, pyelonephritis, pneumonia, endocarditis, prostatitis, osteomyelitis and abscesses or wound infections of any location (Hauptman et al., 1997; Brady and Otto, 2001; de Laforcade et al., 2003). Gram-negative enteric bacteria as well as gram-positive bacteria such as *Streptococcus canis* are reported to be involved in canine septic processes (King, 1994; Brady and Otto, 2001). One study on dogs following surgery for gastric dilation volvulus recognized that complications, such as the development of post-operative hypotension, peritonitis, sepsis, disseminated intravascular coagulation (DIC) and arrhythmia contributed to an increased risk of death (Beck et al., 2006). Such complications are reported in human studies and are characteristic for the progression of SIRS to severe sepsis and organ dysfunction (Johnson, 2004). The occurrence of left ventricular dysfunction in dogs with systemic inflammation and sepsis, which had no prior history of primary cardiac dysfunction has also been described. The authors hypothesize that not only does ischaemia and hypoxaemia lead to insufficient myocardial oxygenation but that the flood of NO and oxygen free-radical impede cardiac myocontractility (Nelson and Thompson, 2006). Organ dysfunction has also been investigated in regards to the occurrence of haemostatic alterations in septic dogs (de Laforcade et al., 2003). Although little has been investigated directly regarding the effect of SIRS on canine renal function, one author does report that renal failure is a serious complication in bitches with pyometra (Zaragoza et al., 2004) and another study which retrospectively investigated the occurrence of acute renal failure in dogs, recognized critical illness as a contributing factor (Behrend et al., 1996).

2.5 Renal parameters and urinary marker proteins for the detection and classification of proteinuria in dogs

2.5.1 Plasma creatinine and the glomerular filtration rate (GFR)

Due to its easy and quick determination, plasma creatinine is traditionally the most commonly investigated parameter in veterinary practice for the initial evaluation of renal function (Elliott, 2006). Whilst creatinine is a non-protein-nitrogen product rather than an actual protein, it is one of the main markers for renal function (Finco and Duncan, 1976). It is produced under normal circumstances in constant amounts as creatine, a compound of muscle cell metabolism, which is then degraded to creatinine (Finco and Duncan, 1976). Creatinine is readily cleared over the glomerulus but is neither secreted nor absorbed in canine kidney tubules (DiBartola, 1980; Braun et al., 2003). A proportion of kidney tissue as large as 75% has to be damaged before elevated creatinine concentrations can be detected, making plasma creatinine a late marker for kidney malfunction (Finco and Duncan, 1976).

Alternatively to the mere plasma creatinine concentration, the state of renal function can be evaluated through the glomerular filtration rate (GFR). The GFR is estimated through the clearance rate of substances, such as creatinine that have unhindered filtration, but are neither reabsorbed nor excreted by the tubule cells (Engelhardt and Breves, 2000). Recent efforts have been made to establish a method for measuring the GFR in routine veterinary practice. This method of modified exogenous creatinine clearance, involves the bolus application of a pre-defined amount of exogenous creatinine. Its subsequent measurement in plasma over time is representative of its clearance through the kidneys (Höchel et al., 2004). The GFR and the creatinine concentration in plasma have a hyperbolic relationship with each other, meaning that when the GFR is reduced, creatinine concentration in plasma increases. Up to a point therefore, the creatinine concentration itself does provide valuable information on the state of the GFR (Finco and Duncan, 1976; DiBartola, 1980; Silbernagl and Despopolous, 2001). Creatinine values for dogs as used in the Clinic for Small Animals at the FU Berlin, based on recommendations from the International Renal Interest Society (IRIS) and the European College of Veterinary Internal Medicine (ECVIM) congress in 2006 (Elliott, 2006), are shown in table 5.

Table: 5 Reference values for the use of creatinine levels for the evaluation of renal function in dogs, (modified from Elliott, 2006)

Creatinine (µmol/l)	**Interpretation**
53 - 106 (dogs < 20 kg) 53 - 124 (dogs > 20 kg)	Normal, non-azotaemic
106/124 - 179	Mild renal azotaemia
180 - 439	Moderate renal azotaemia possible presence of extra-renal signs
> 440	Severe renal azotaemia usual presence of extra-renal signs

Azotaemia is the increased presence in blood of nitrogen-containing compounds such as creatinine and urea. Pre-renal azotaemia occurs when renal perfusion is decreased due to hypovolaemia (shock, haemorrhage, dehydration) or cardiac insufficiency. In renal azotaemia the glomerular filtration is decreased due to the loss of functional nephrons resulting in insufficient renal filtering of nitrogen-containing compounds. The urine specific gravity (USG) is an important differentiating parameter between pre-renal and renal azotaemia as the concentrating ability of the kidneys is impaired in the latter. A USG of >1.030 is common for pre-renal azotaemia and isosthenuric urine (USG 1.008 – 1.012) is characteristic of renal azotaemia. Based on the USG alone though, differentiation is difficult when the USG is between 1.012 and 1.030. Post renal factors such as a rupturing or obstruction of the lower urinary tract can also lead to azotaemia (Ettinger and Feldman 2005; Kraft and Dürr, 2005; IRIS Guidelines 2010). Table 6 depicts various alterations in selected biochemical and urinary parameters, depending on the origin of the azotaemia.

Table 6: Parameters used for differentiating between pre-renal, renal and post-renal azotaemia (modified from Ettinger and Feldman 2005 and Kraft and Dürr 2005).

Parameter	Pre-renal	Renal	Post-renal
Serum creatinine	↑	↑	↑
BUN	↑	↑	↑
PCV	↔ / ↑	normal (ARF) normal to ↓ (CRF)	normal
UPC	normal	↑	↑
USG	> 1030	< 1030	variable
BUN post i.v. fluid infusion	rapid decrease	little change or possible normalisation if a pre-renal factor also involved	

↑ = above reference range, ↓ = below reference range, ↔ = within reference range
ARF- acute renal failure, CRF - chronic renal failure

2.5.2 Urinary protein to urinary creatinine ratio (UPC)

It is current practice in both veterinary and human medical fields to express proteinuria as a unit less urinary protein to urinary creatinine ratio (UPC) (Barsanti and Finco, 1979; Grauer et al., 1985; Waller et al., 1989; Lulich and Osborne, 1990; Lees et al., 2005). This is due to the fact that the urine protein concentration at a specific point in time is variable and is influenced by the total urine volume, which is also partly dependent on the hydration status. In a small urine volume, the protein concentration appears higher than if it was diluted by the presence of a higher volume of fluid. Despite this however, the relationship of protein to creatinine in an "untimed spot sample" remains constant. The UPC can also be used to confirm a positive semi- quantitative test and provide an indication of the magnitude of the proteinuria (Lees, 2004). Although a 24-hour urine collection would provide an absolute value for proteinuria, such a procedure is understandably not feasible in the daily veterinary practice. Proteinuria as defined by the American College of Veterinary Internal Medicine (ACVIM) consensus statement from 2004 (Lees et al., 2005) with recent amendments from International Renal Interest Society (IRIS 2009) is shown in table 7.

Table 7: Interpretation of canine UPC (modified from Lees et al 2005 and IRIS 2009)

UPC value	Interpretation
< 0.2	normal
0.2 - 0.5	borderline proteinuric proteinuria is determined by persistence of the increased UPC
> 0.5	abnormal, indicates proteinuria
> 2	grossly abnormal, indicates overt proteinuria

2.5.3 Urinary Albumin

Albumin is the most abundant plasma protein in dogs and humans, accounting for about 60% of proteins circulating in the blood. It is produced in the liver and functions as a transport protein and a buffer in plasma and due to its high concentration, is largely responsible for upholding the colloid osmotic pressure (Engelhardt and Breves, 2000). It has a molecular weight of 69 kDa and belongs therefore to the group of middle molecular weight proteins (MMW). It has an absolute radius of 3.6 nm (Brenner et al., 1978; Waller et al., 1989) or an effective radius of 7.5 nm (Gekle, 1998). Despite these size attributes, which theoretically should prevent the passage of albumin passage through the glomerular filter, its high heamodynamic force ensures its presence in the ultrafiltrate. In humans approximately 0.05-0.1% of plasma albumin reaches the ultrafiltrate of which 99% is reabsorbed through

receptor-mediated endocytosis (Gekle, 1998). Although albumin is a ligand for both cubilin and megalin, the importance of cubilin in albumin reabsorption is emphasized by the albuminuria observed in dogs with cubilin defects (Verroust, 2002). In dogs, as with humans, this minimal albumin presence still represents 40-60% of the total urinary proteins (Lulich and Osborne, 1990; Zaragoza et al., 2004). When present in higher than normal amounts, albumin is the most common marker for proteinuria of a glomerular nature (DiBartola, 1980).

Microalbuminuria (MA) is the presence of a higher than normal level of albumin, which is however not yet detectable through means of conventional dipsticks. Such dipsticks are sensitive for concentrations of albumin greater than 300 mg/l (Vaden, 2004). Urinary albumin levels for dogs are as follows in Table 8.

Table 8: Interpretation of canine urinary albumin levels, (modified from Jensen et al 2001, Lees 2004, Vaden 2004, Grauer 2005)

Urinary albumin concentration mg/l	**Interpretation**
< 10	negligible, within normal expectancy
10 - 300	microalbuminuria
> 300	overt albuminuria/proteinuria

Especially when dealing with spot urine samples, some authors have examined a urinary albumin to urinary creatinine ratio (UAC) as an alternative for the definition for microalbuminuria (Thorevska et al., 2003; Lees, 2004; Lees et al., 2005). In the ACVIM forum consensus statement, on the assessment and management of proteinuria in dogs and cats (Lees et al, 2005), a UAC greater than 30 mg/g is reported to represent MA. In humans, overt proteinuria has been defined by Thorevska et al (2003) as a UAC greater than 300 mg/g.

2.5.4 Retinol-binding protein

Retinol-binding protein (RBP) is a low molecular weight protein (21 kDa), and a member of the lipocalin gene family. RBP is synthesized primarily in the liver, functions as the main carrier protein for vitamin A and occurs in plasma primarily in the holo-form of a ternary retinol-RBP-TTR complex through its binding with retinol and transthyretin (TTR) and also in a TTR-unbound apo-RBP form (Raila et al., 2003). Although under physiological conditions, apo-RBP is freely filtered in the glomerulus, virtually all RBP should be reabsorbed from the ultrafiltrate through megalin mediated endocytosis, so that end urine is virtually free of RBP (Raila et al., 2000). RBP is therefore a recognized marker for proximal tubular injury and can facilitate early detection of tubular malfunction as its levels can be slightly raised long before

recognizable changes in other parameters, such as serum creatinine or overt proteinuria have occurred (Bernard et al., 1987). Monitoring of urinary RBP excretion can therefore provide a means of assessing the risk of tubular necrosis or progressive destruction of tubules (Bernard et al., 1987). RBP is particularly a marker for the state of megalin function, as has been shown through studies on megalin-knockout mice who have LMW proteinuria, with particularly high RBP excretion (Christensen et al., 1999; Christensen and Birn, 2001).

3 Material and methods

3.1 Material

3.1.1 Patients and control dogs

The patients for this study were presented for treatment at the Small Animal Clinic, Freie Universität Berlin (FU Berlin), Germany between April 2004 and October 2005. The dogs in the patient group (n=39) demonstrated two 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 as described previously (Hauptman et al., 1997; Brady and Otto, 2001; de Laforcade et al., 2003). The dogs in the patient group were presented at various times during regular clinic and emergency opening hours and were initially treated according to standard practice for emergency patients (e.g. fluid replacement therapy, antibiotics etc). Dogs for the control group (n=15) were chosen randomly from animals presented at the clinic for the purpose of blood donation or routine examination. Furthermore, based on history, clinical, haematological, biochemical and urinary examinations, these dogs were deemed to have had neither previous history nor any current clinical signs of renal impairment.

3.1.2 Collection and preservation of samples

Urine was collected mostly through midstream catching of voluntary urination and cystocentesis or catheterization were used in a few cases. Whenever possible and depending on the animal's outcome, blood and urine were collected from each of the diseased animals at 3 designated points in time, namely at admission or within 24 hours thereof (day 0) and on the following two days thereafter (day 1, 24 hours after initial sample and day 2, 48 hours after initial sample). The control dogs were examined once, on presentation. Urine was divided into 1 ml aliquots and then frozen at -80℃ until needed for testing. Urine specific gravity was measured using a handheld refractrometer. Blood was obtained from the *V. cephalica*, *V. saphena* or *V. jugularis* and collected in EDTA vials for the complete blood count and heparin vials for plasma and immediately examined following standard procedures. The CELL-DYN 3500 (Abbott Diagnostika, Hofheim-Wallau, Germany) was used for haematological examination, a manual differential blood count was performed and the KONELAB 30i (Thermo Electra, Dreieichen, Germany) was used for blood chemistry.

3.1.3 Parameters examined

Urine was examined for the parameters total protein content, creatinine, specific gravity (USG) and the concentrations of albumin and retinol-binding protein. An electrophoresis separation of urine samples was undertaken to evaluate the presence of further protein patterns indicating glomerular or tubular lesions. For diagnostic purposes and for thorough investigation of the underlying illness, standard blood biochemistry parameters and the complete blood cell count were examined and depending on the suspected underlying illness, further diagnostic tests such as diagnostic imaging, microbiological and immunological testing and cytology were performed. These results are not included in the current study.

3.2 Methods

3.2.1 Measurement of urinary protein with the Bradford method

Urinary protein was measured using the Bradford method. This method is based on the binding of proteins in an acidic solution to Coomassie brilliant blue G-250, a triphenylmethan dye. The absorption maximum of the reagent solution alone is 465 nm, which increases to 595 nm when proteins are bound. Using spectrophotometric determination of absorbance, the increase in adsorption at 595 nm allows measurement of the protein concentration in the solution.

For the Bradford solution, 100 mg of Coomassie G 250 (Sigma, Steinheim) and 50 ml of 95% ethanol were mixed in a glass flask using a magnet mixer. The solution was transferred to a 1 l flask, 100 ml of 85% phosphoric acid was added and the solution was diluted with distilled water to a final volume of 1 l. Before initial use, the solution was filtered through filter paper. The standard protein solution was prepared using bovine crystalline albumin A (Serva, Heidelberg) which was dissolved in distilled water to give an initial concentration of 5 mg/ ml. This was diluted proportionally 1:2, 7 times to give a total of 8 solutions ranging between 5 mg/ml and 0.039 mg/ml for a standard concentration curve. 100 µl of each urine sample or each standard dilution were mixed with 900 µl of the Bradford solution. For a reagent blank, 100 µl of distilled water was also mixed with 900 µl of the Bradford solution. 200 µl of each sample, standard or blank was applied in triplicate to wells of a 96-well microtiter plate (Greiner Bio-One, Frickenhausen). Absorbance of the samples was immediately evaluated at a wavelength of 570 nm for 3 seconds in a spectrometer (Microplate reader Model 680 XR, Bio-Rad, Munich) using Microplate manager version 5.2 software. The protein concentrations were calculated using the machine specific software.

3.2.2 Measurement of urinary creatinine with the alkaline picrate method

Creatinine concentration in urine was measured following the alkaline picrate method with protein denaturation and using a kit from Randox Laboratories (Crumlin, UK). The reagent solution from Randox comprised 177 µmol/l creatinine standard CR 511, 35 mmol/l picric acid and 1.6 mmol/l sodium hydroxide. Urine samples were diluted 1:50 with distilled water and the sealed vials were placed in boiling water for 5 minutes for protein denaturation. Distilled water was used as a blank. Wells of microtiter plates (Greiner Bio-One, Frickenhausen) were filled in triplicate with 50 µl of either sample, standard or blank. 100 µl of reagent and 50 µl of trichloric acid (TCA) were added to each well and plates were incubated at 25°C for 20 minutes. The colour intensity was read in a spectrometer at 490 nm using the microplate reader and software from BioRad as used for the protein determination.

3.2.3 Enzyme-linked-immunoabsorbend-assay (ELISA) of urinary albumin and urinary RBP

3.2.3.1 Solutions used for ELISA

Table 9: Solutions used in ELISA for the determination of RBP and albumin in canine urine

Solution	Contents
PBS washing solution	Phosphate buffered saline. 10 mM phosphate buffer, 150 mM sodium chloride, pH 7.4
PBS-TWEEN washing solution	10 mM phosphate buffer, 150 mM sodium chloride, Tween containing 0.05% bovine serum albumin (BSA V code nr. 11930, Serva, Hamburg), pH 7.4
Carbonate buffer	50 mM, pH 9.6
O-phenylenediamine Dihydrochloride	OPD (Sigma, Diesenhofen) diluted to 3.7 mM in citric acid buffer
Citric acid buffer	50 mM disodium phosphate-25 mM citric acid buffer pH 5.2

3.2.3.2 ELISA for retinol-binding-protein (RBP)

The urinary concentration of RBP was determined by ELISA as described previously by Topping et al (1986). Microtiter plates (Costar, USA) were washed 4 times with PBS washing solution in an automatic washer (Columbus Washer, Tecan, Crailsheim). Wells were then applied with 50 µl of rabbit anti-human serum-RBP IgG (A 0040, Dako Cytomation, Denmark) diluted in carbonate buffer to a final concentration of 10 µg/ml. The plates were incubated for

2 hours at 37℃ with constant shaking and then over night at 4℃. Plates were rewashed with PBS-Tween. The standard solution, using the control serum N Protein Standard/-Standard SL OQIM 13 (Dade Behring, Marburg) was diluted with PBS/0.1% BSA to produce a standard row with 7 dilution levels ranging from 45 to 2 ng/ml. Urine samples were diluted 1:10 with PBS/0.1% BSA. 50 µl of each diluted urine, standard dilution and PBS/0.1% BSA as a control were applied in triplicate to the wells and incubated for 1 hour at 37℃ with constant shaking. Plates were subsequently washed 4 times automatically with PBS-Tween. 50 µl of peroxidase-conjugated anti-RBP IgG (P0304, Dako) diluted 1:2000 with PBS/0.1% BSA was applied to each well as a cross-reacting antibody and the plates were incubated at 37℃ with constant shaking for 1 hour before a final washing with PBS-Tween. The colour reaction was achieved through adding 100 µl OPD-citric acid solution with 0.012% hydrogen peroxide and incubating at 25℃ for 15 minutes before stopping the reaction with 50 µl 1M sulphuric acid. The absorption was measured at 490 nm using the microplate reader (Bio Rad) as already described. The standard dilution row was plotted as a half logarithm against the absorption and a linear calibrated curve was achieved through the use of regression analysis. RBP concentrations that fell within this range were calculated using the microplate software (Bio-Rad).

3.2.3.3 ELISA for albumin

An ELISA for the determination of urinary albumin was performed following the recommendations and instructions from Bethyl Laboratories Inc. USA. Anti-dog-albumin (A40-113A, Bethyl) was diluted 1:1000 in carbonate buffer and 50 µl was applied to the wells of a microtiter plate (Costar) and the plates were incubated at 37℃ under constant shaking for 1 hour and then at 4℃ over night. Plates were subsequently washed 4 times with PBS-Tween and filled with 200 µl of the blocking solution and then incubated at 37℃ without shaking for 5 minutes. The blocking solution, Super Block buffer Dry Blend in Tris buffered saline (Pierce, USA) was prepared fresh daily following the manufacturer's instructions. After blocking, the plates were once again rinsed with PBS-Tween. Canine albumin (Sigma Aldrich, USA) in an original concentration of 10 mg/ml of PBS solution was further diluted in PBS solution until 8 dilution levels were achieved ranging from 100 µg/l to 0.781 µg/l. Depending on the suspected level of albuminuria (based on the sample's UPC) urine samples were diluted 1:1000, 1:2000 or 1:5000 in saline solution. 50 µl of standards, samples and saline solution as a blank were applied in triplicate to the plate wells and incubated for 1 hour at 27℃ under constant shaking. After washing with PBS-Tween the detection antibody was applied. Dog albumin HRP-conjugated antibody (A40-113P, Bethyl) was diluted 1:20 000 in saline solution and 50 µl of this dilution was applied to each well and

the same incubation and rinsing processes were once again followed. To obtain the colour reaction, 100 µl of the OPD-citric acid solution with 0.012% hydrogen peroxide was applied to each well and plates were incubated at 24℃ for 15 minutes. The reaction was stopped through adding 50 µl 1M sulphuric acid. The absorption was measured at 490 nm using the microplate reader (Bio-Rad) as already described.

3.2.4 Sodium dodecyl-sulfat-polyacrylamid-gel electrophoresis (SDS-PAGE)

SDS-PAGE allows the separation of proteins depending on their molecular weight, which determines how quickly each protein can migrate through a porous, sieve-like polyacrylamid gel matrix under the influence of an electric field. For this purpose it is necessary that all proteins exhibit the same charge/mass relationship so that the only deciding factor is the difference in molecular weight. Through the addition of the detergent SDS (Sodiumdodecyl-sulfat, 1.4 mg/ mg protein) protein denaturation is achieved and all proteins are present as negatively charged SDS-protein complexes. Electrophoresis was performed following the method from Laemmli (1970).

The 12% polyacrylamid separation gel was made from 150 mM Tris-HCl, ph 8.8 with 0.1% w/v SDS, 121 g/l acrylamid/N,N`-methylenbisacrylamid, 0.76 ml/l tetramethylethylendiamin (TEMED), 0.5 g/l ammoniumpersulfat and distilled water. The 3% collecting gel was made from 50 mM Tris-HCl, pH 6.8 in 0.1% w/v SDS, 39.9 g/l acrylamid/bisacrylamid, 1 ml/l TEMED, 0.5 g/l ammonium persulfat and distilled water. For dilution of the samples a buffer solution of 125 mM Tris, 20% glycerol, 2% SDS and bromophenol blue was used. Urine aliquots were diluted 1:1 with buffer solution or 1:10 when the Bradford protein measurement method showed samples to be extremely high in protein. The aliquots were mixed and placed in a water bath at 95℃ for 5 minutes for co mplete denaturation of the proteins.

Five µl of a commercially available molecular weight standard (SDS-PAGE molecular weight standard, Low Range; Bio-Rad) was placed in the first slot of each gel followed by seven slots each with 15 µl of the prepared samples. The electrophoresis took place in the device Mini-Protean II (Bio-Rad) with 1 l electrode buffer (25 mM tris, 192 mM Glycin and 0.1% SDS, pH 8.3) under the constant current of 50 mA for 1 hour. On completion, the gels were stained with silver nitrate stain following the method from Heukeshoven and Dernick (1988) and examined using the scanner system, ChemiDoc XRS and the software Quantity One from Bio-Rad. The molecular mass of the urinary proteins was evaluated by comparing with the Low Range molecular weight standard which included the following bands: 14.4 kDa, lysozyme; 21.5 kDa, trypsin inhibitor; 31 kDa, carbonic anhydrase; 45 kDa, ovalbumin; 66.2 kDa, serum albumin; 97.4 kDa, phosphorylase b. The technique of visual inspection, as has

been reported by previous authors was used to classify proteinuric patterns (Stierle et al., 1990; Yalcin and Cetin, 2004).

3.2.5 Methods of statistical evaluation

The collected data was analyzed using the program SPSS 11.0 (SPSS GmbH Software, Munich). Mean, median and range for each parameter and group were calculated. The differences between the groups were analyzed using the Mann-Whitney test for non-parametric, unpaired data and trends and prognosis were analyzed using binary logistic regression. Differences were considered significant at a p value of equal or less than 0.05.

4 Results

4.1 Characteristics of the diseased and healthy groups

The diseased group was comprised of 39 dogs with SIRS. They were initially presented for a variety of reasons, the main symptoms being reported as inappetence (n=10), lethargy (n=8), vomiting (n=7), lateral recumbency (n=5), purulent/haemorrhagic vaginal discharge (n=3) defecation/urination disturbances (n=2), polyuria/ polydipsia (n=2), diarrhoea (n=1) and respiratory distress (n=1). The diagnosed underlying diseases or conditions are listed in Table 10. The diseased group included 18 entire and 2 spayed bitches, 15 entire and 4 neutered males. The most frequently represented breeds were German shepherd (n=6) and mix-breed (n=6) and in total 21 different breeds were represented. Weights ranged from 8-50 kg with a median of 29 kg. The diseased dogs were significantly older than the healthy dogs ($p<0.001$) with a median age of 8 years (range, 1 to 13 years) compared to a median of 4 years for the healthy dogs (range 1 to 10 years). The healthy control group (n=15) included 3 entire and 3 spayed bitches, 3 entire and 6 neutered males.

Table 10: Underlying diseases for the group of diseased dogs (n=39)

Underlying disease	Number of dogs
Immune mediated haemolytic anaemia (IMHA)	11
Pyometra	7
Peritonitis	5
- ruptured bowel due to foreign body (2)	
- peri-anal hernia with ruptured bladder (1)	
- ruptured gall bladder (1)	
- perforated bowel due to neoplasia (1)	
Skin abscess	4
Prostatic abscess	3
Acute gastritis	1
Fever of unknown origin	1
Haemometra	1
Mesenteric infarction	1
Necrotizing tonsillitis	1
Parvovirosis	1
Salmonellosis	1
Splenic neoplasia	1
Underlying disease undiagnosed	1

4.2 Outcome of the patients

The median stay in the clinic for the diseased dogs was 5 days and ranged from 1 to 14 days. Of the 39 diseased dogs, 24 remained in the clinic for at least the duration of the test period so that 24 dogs provided urine samples on both days 0 and 2. Of the 15 dogs that did not provide day 2 values, 10 died before day 2 and 5 were discharged. Dogs that survived until discharge were considered survivors. The number of non-survivors was 13 (33%) of which 4 dogs died naturally, whilst the remaining 9 non-survivors were euthanized. The mortality rate for the patients in this study was comparable to mortality rates described by other authors in similar studies (range 30-50%) (Hauptman et al., 1997 ; de Laforcade et al., 2003; Rau et al., 2007). The underlying diseases of the non-survivors were IMHA (n=4), peritonitis (n=2), pyometra (n=2), skin abscess (n=2), fever of unknown origin (n=1), splenic neoplasia (n=1) and underlying disease undiagnosed (n=1). Of the 13 non-survivors, 10 had died or were euthanized by day 2 of the study period, 2 were euthanized on day 5 and 1 dog was euthanized on day 6. The decision for euthanasia was made based entirely on veterinary medical grounds with the intention of relieving an animal of further pain and suffering in a case where the prospects for survival and recovery were considered immensely slim. In none of the cases was this decision reached due to financial concerns of the owner. Whether an animal was euthanized or died naturally will therefore be regarded as the same for the purpose of analysis in this study.

4.3 Urine parameters for the detection of glomerular and tubular dysfunction

4.3.1 Total urinary protein content

The urinary protein content for the diseased dogs on day 0 ranged from 3.6-1361 mg/dl with a median protein excretion of 71.8 mg/dl. Healthy control dogs had a protein excretion ranging from 5.5-103 mg/dl with a median of 18.7 mg/dl. The total urinary protein excretion of the diseased dogs was therefore significantly higher ($p<0.001$) than that of the healthy controls (figure 3). All but one of the healthy dogs had urinary protein levels well below the suggested limit for physiological proteinuria of 65 mg/dl (Barsanti and Finco, 1979). Dog 42, (see Table 21) a sexually intact male had a urinary protein level of 102 mg/dl. Twenty-two of the sick dogs (56%) had urinary protein levels above 65 mg/dl. No significant difference was evident in protein excretion between survivors and non-survivors with median excretion levels of 74.3 mg/dl (3.6-592 mg/dl) and 67 mg/dl (18.8-1361 mg/dl), respectively.

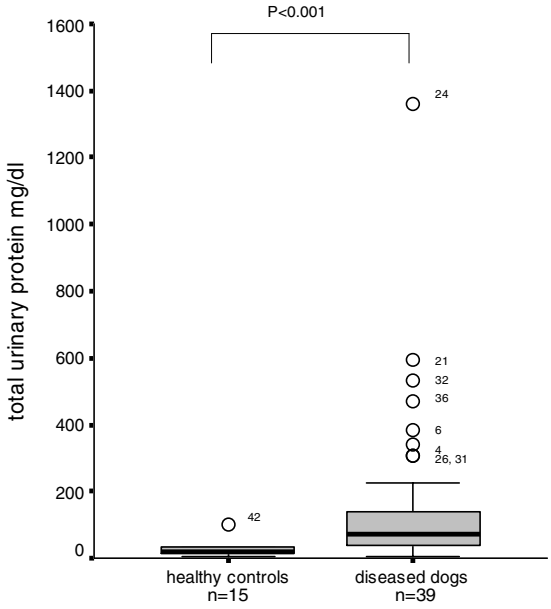

Figure 3: Total urinary protein content on day 0.

4.3.2 Urinary creatinine

Urinary creatinine excretion for the diseased dogs was 8.2-520 mg/dl, the median being 88.8 mg/dl and for the healthy dogs the range covered 80.2-375 mg/dl with a median of 228 mg/dl. Although there are currently no established values for minimum or maximum creatinine levels in canine urine, these results are in comparable proportion to those recorded by Forterre (2003), where healthy controls had a median creatinine excretion of 354 mg/dl as opposed to 47.5 mg/dl for dogs with renal insufficiency. In the current study urinary creatinine was measured for the calculation of ratios with total protein, RBP and albumin in order to compensate for the diluting or concentrating effect of differing total urine volumes.

4.3.3 Urinary protein: creatinine ratio (UPC)

The median UPC value for the diseased dogs was 0.65 and a range from well within normal up to overt proteinuria (0.09-7.94) was covered. For the control dogs a median of 0.09 was recorded (range 0.03-0.34) so that a highly significant difference was evident, as depicted in Figure 4. The diseased dogs showed therefore a significantly higher loss of protein in urine, both as an absolute amount and in relationship to creatinine excretion as expressed through the UPC. Twenty-seven diseased dogs (69%) had a UPC higher than 0.5. Of these dogs, 11 had a UPC between 0.5 and 1.0 and 16 dogs had overt proteinuria as indicated by a UPC exceeding 1.0 (5 dogs) or 2.0 (11 dogs). Only two cases where the absolute protein excretion was above normal were associated with a normal UPC. Conversely, 6 dogs

showed a UPC >0.5 with a normal absolute protein excretion. Once again, no significant difference was evident between survivors (median 0.95, range 0.9-7.94) and non-survivors (0.61, 0.11-4.80) on day 0.

When comparing the UPC values recorded during the course of the examination period, the UPC values from day 0 were significantly higher than those recorded on day 2 (Wilcoxon $p<0.05$). Of the 24 SIRS dogs where urine samples could be obtained during the 48-hour study period, 17 had decreasing UPC values and 7 demonstrated UPC increases. The dogs with initial UPC values in the normal reference range (n=8) remained in this range, apart from one dog whose UPC increased from 0.26 to 0.53 on day 2. Of the dogs whose initial UPC was greater than 0.5, day 2 values were also available for 16 dogs. Twelve of these dogs had decreasing values, whilst 4 dogs had increasing UPC values from day 0 to 2.

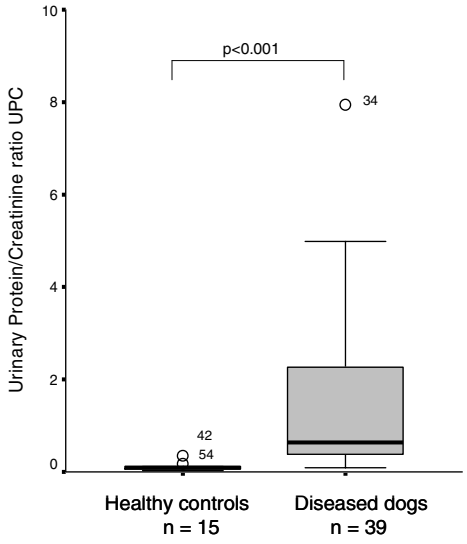

Figure 4: UPC values on day 0 of clinical admission.

4.3.4 Urinary content of retinol-binding protein

The urinary RBP excretion levels on day 0 for the diseased dogs (median 122 µg/l, range 29–205 µg/l) were significantly higher than those for the healthy dogs (median 36.5 µg/l, range 27-67 µg/l) as shown in Figure 5. In order to acknowledge the effect of varying urine volume on urine protein concentration, the ratio of urinary RBP to urinary creatinine (URBPC) was examined. For the diseased dogs a median of 120 µg/g and a range of 22.6–788 µg/g was calculated in comparison to a median of 18 µg/g and range of 9.6–33.8 µg/g for the healthy dogs, indicating once again a highly significant difference ($p<0.001$). On day 0, survivors (n=26) had a median URBPC of 147 µg/g (22.6–768) and non-survivors (n=13) a

median of 85.6 µg/g (29.1–537). On day 2 the median URBPC for survivors (n=21) was 140 µg/g (28-470 µg/g) and for non-survivors (n=3) 305 µg/g (155-521 µg/g). In both cases no significant difference was evident.

Twenty-four diseased dogs were tested for urinary URBPC on days 0 and 2. Eleven dogs had decreasing values from day 0 to day 2 and 13 dogs demonstrated an increase in URBPC from day 0 to day 2. Of these animals, 4 dogs exhibited an initial increase in URBPC from day 0 to day 1 before a decrease in RBP excretion from day 1 to day 2 whereby the URBPC on day 2 was still higher than the initial days 0.

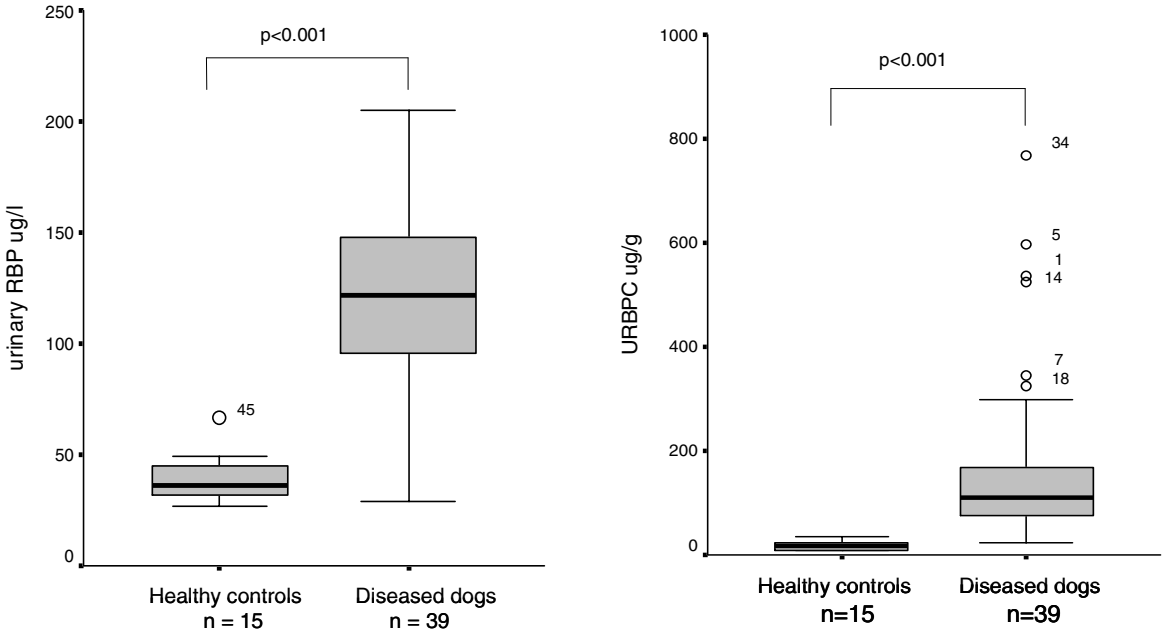

Figure 5: Urinary RBP levels on day 0 expressed as RBP (µg/l) and URBPC (µg/g)

4.3.5 Urinary content of albumin

The median urinary albumin excretion for the diseased dogs was 32.8 mg/dl (range 2.4-530 mg/l) and for the healthy dogs, 2.35 mg/l (range 0.42-16.5 mg/l). When expressing urinary albumin excretion as a ratio to urinary creatinine (UAC), the median for the diseased dogs was 46.1 mg/g (range 3.1-1338 mg/g) and 1.9 mg/g for the healthy dogs (range 0.2-8.3 mg/g). The albumin excretion of the diseased dogs was therefore significantly higher than the healthy dogs, both as an absolute amount and as a ratio to creatinine (figure 6). When considering MA as a urinary albumin concentration > 10 mg/l (Lees, 2004), 32 of the diseased dogs (82%) had evidence of MA. When based on a UAC > 30 mg/g (Lees et al., 2005), 25 of the diseased dogs (64%) had MA on day 0, a discrepancy existing for 7 dogs whose absolute albumin values were only narrowly increased and whose UPC values were

normal or only slightly above 0.5. Five dogs (dogs 6, 9, 30, 34 and 38) exhibited UAC levels above 300 mg/g with 523, 359, 397, 1338 and 317 mg/g respectively and can therefore be described as having macroalbuminuria or overt proteinuria (Thorevska et al., 2003). The high level proteinuria of these dogs was confirmed by their respective UPC values of 4.98, 3.47, 1.5, 7.97 and 1.99. In contrast, all 15 healthy dogs had UAC levels < 30 mg/g.

The median UAC on day 0 for survivors (n=26) was 73.9 mg/g (3.1-1338) and 28.6 mg/g (5.6-225) for non-survivors (n=13). On day 2 survivors (n=21) had a median UAC of 17.2 mg/g (1.7-916) and non-survivors (n=3) 158 mg/g (34.2-301). No significant difference was apparent for the two groups in regards to urinary albumin excretion on either day.

Twenty-four dogs were tested for urinary albumin excretion on days 0 and 2. As occurred with the UPC, serial measurements of albumin throughout the examination period demonstrated a declining trend. The Wilcoxon test comparing the values from days 0 and 2 showed a significant difference between the two days ($p<0.05$). Of the 24 diseased dogs for which values were available on days 0 and 2, 14 dogs (63%) had decreasing levels, 9 dogs (33%) exhibited a higher level of urinary albumin excretion on day 2 than on day 0 and 1 dog maintained its normal levels. Eleven of the dogs with decreasing albumin had recovered from MA to a normal level by day 2, whilst 4 dogs still had MA on day 2.

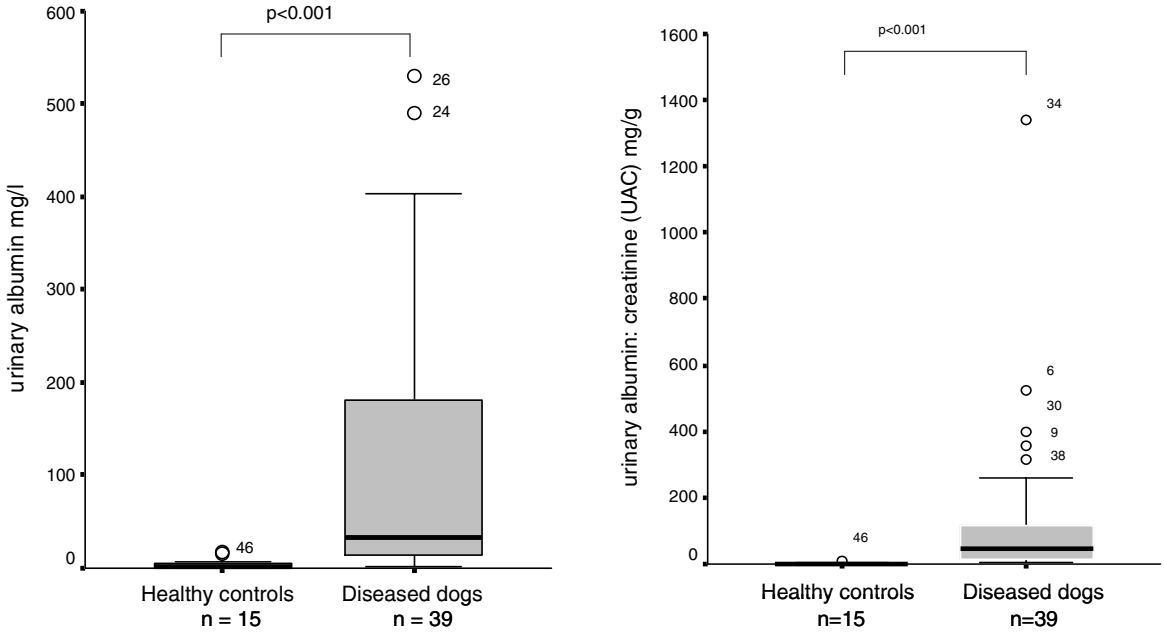

Figure 6: Urinary albumin levels on day 0, expressed as urinary albumin and UAC.

4.3.6 Correlations between the variables found in urine

When examining the relationships between the urinary parameters, the Spearman´s rank correlation test showed a positive correlation between all urinary parameters, with especially strong correlation between total urinary protein and UPC as well as total urinary protein and albumin with rho values of 0.792 and 0.718 respectively. Similarly, there were strong positive correlations between the UPC and UAC (0.776), UPC to URBPC (0.576) and UAC to URBPC (0.583). In each case the p value was <0.001.

Table 11: Positive correlations between the individual urine parameters

Relating parameter		**Correlation** (rho)	**Significance** (p)
Total protein	UPC	0.792	p<0.001
Total protein	Albumin	0.718	p<0.001
Total protein	RBP	0.580	p<0.001
UPC	RBP	0.612	p<0.001
UPC	URBPC	0.576	p<0.001
UPC	Albumin	0.749	p<0.001
UPC	UAC	0.776	p<0.001
RBP	URBPC	0.483	p<0.01
RBP	Albumin	0.626	p<0.001
Albumin	UAC	0.869	p<0.001
URBPC	UAC	0.583	p<0.001

weak positive correlation rho 0 - 0.5, strong positive correlation rho 0.5 - 1

4.4 Urinary protein pattern by SDS-PAGE

An SDS-PAGE of urine samples was conducted for each dog and wherever possible, the series of urine samples from days 0, 1 and 2 were chronologically examined. For each of the healthy controls, 1 urine sample was examined. Table 12 shows the molecular weight ranges and the frequency of appearance of the bands in each weight range in the urine of healthy control dogs and the diseased dogs. Depending on the presence of bands at specific molecular weights and following the molecular weight groupings as used by Yalcin (2004), a pattern containing MMW proteins (60-80 kDa) was defined as selective glomerular, MMW and HMW proteins (>60 kDa) is defined as non-selective glomerular, and the presence of LMW proteins (<60 kDa) depicts proteinuria of tubular origin. Mixed proteinuria was characterized by the presence of LMW, MMW and HMW proteins whilst a pattern containing only a single band in the range 60-70 kDa (albumin) was defined as physiological (Yalcin and Cetin, 2004).

Table 12: Frequency of dogs, which had bands of each molecular weight range, as, detected by SDS-PAGE analysis.

MW ranges kDa	**Diseased** (n=39) % of dogs	**Healthy** (n=15) % of dogs
> 110	27	-
100-110	7	-
80-100	70	-
60-70	92	78
50-60	40	-
40-50	37	-
30-40	48	-
20-30	57	21
10-20	77	43

4.4.1 Urinary protein patterns from healthy control dogs

Three different bands with molecular masses of 69-70 kDa, 20-30 kDa and 10-20 kDa occurred in urine from the healthy control animals. Four dogs exhibited no bands, whilst a faint band in the range 60-70 kDa dominated the electrophoresis pattern of the other 11 dogs. Five dogs had only this band. Three dogs, (dogs 42, 53 and 54) all sexually intact males, had the highest number of bands, which occurred with varying distinction at 17, 30, and 60-70 kDa. An example of urine electrophoresis from healthy control dogs, including dogs 53 and 54 is depicted in Figure 7. In total, 55% of the counted bands were in the MMW/HMW range and 45% were of the LMW type.

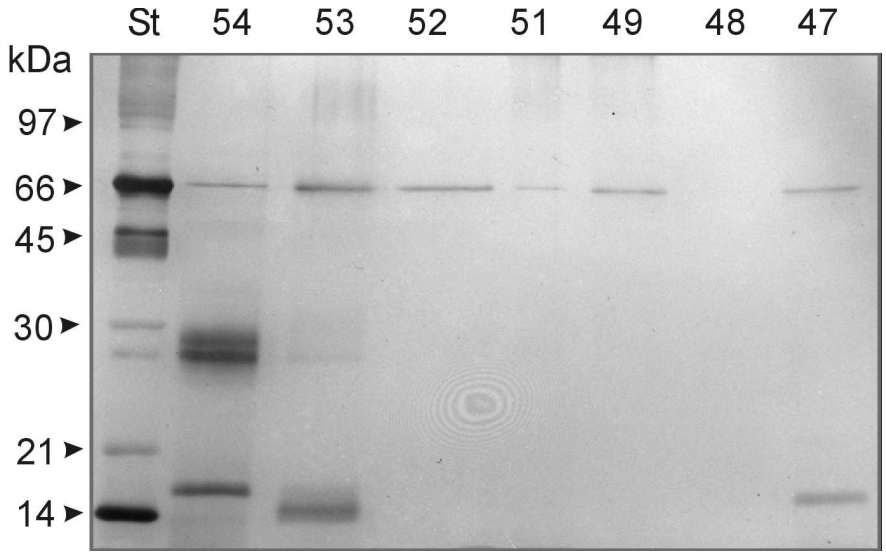

Figure 7: SDS-PAGE of urine samples from the healthy control dogs. Vertical lanes from left to right contain the standard and urine from dogs as numbered.

4.4.2 Urinary protein patterns from diseased dogs

In general, the electrophoresis patterns in urine from the diseased animals were far more heterogenic than those from the healthy counterparts and a total of 11 different bands were observed. As with the healthy dogs though, the presence of a band in the range 60-70 kDa pre-dominated, occurring in 36 (92%) dogs and a further pattern was evident as 23 of these dogs also exhibited a 90-100 kDa protein. Despite this pattern, the distribution of the numbers of bands in the categories MMW/HMW and LMW was very different to that of the healthy animals in that 42% of the observed bands were in the MMW/HMW range and 58% in the LMW range. Based on the presence of bands in the various molecular weight categories in the SDS-PAGE from day 0, the protein pattern of each diseased dog was interpreted as physiological, glomerular, tubular or of mixed nature as presented in table 13.

Table13: Total number and percentage of diseased dogs with each type of proteinuria, based on optical assessment of SDS-PAGE.

	Physiological	Glomerular Non-selective	Tubular	Mixed
Number of dogs (n=39)	8	2	11	18
%	21	5	28	46

A representative sample of SDS-PAGE urinary protein patterns from diseased dogs 26, 27 and 29 is depicted in figure 8. Dog 26 (neutered male) had mixed proteinuria on day 0 with a clear albuminuria (confirmed by the ELISA result of 539 mg/l albumin, a UAC 225 mg/g and a UPC of 1.29). Analogue to the dog's diminishing UPC over the following two test days, (day 1: 0.56 and day 2: 0.39), the density and number of protein bands also decreased so that by day 2, a physiological proteinuria was evident. On day 2 a band was evident only in the range 60-70 kDa. The electrophoresis of urine from dog 27 (entire female) demonstrated a mild selective glomerular proteinuria over all three test days which corresponded to UPC values of 0.6, 0.6 and 0.7, albumin levels of 14, 18 and 16 mg/l and UAC values of 15.2, 18 and 16 mg/g. Dog 29 (entire female) demonstrated a mild, mixed proteinuria, corresponding to its UPC of 0.72, albumin 70 mg/l, UAC 123 mg/g, RBP, 92 µg/l and URBPC 161 µg/g. Dogs 26 and 27 were survivors, dog 29 died.

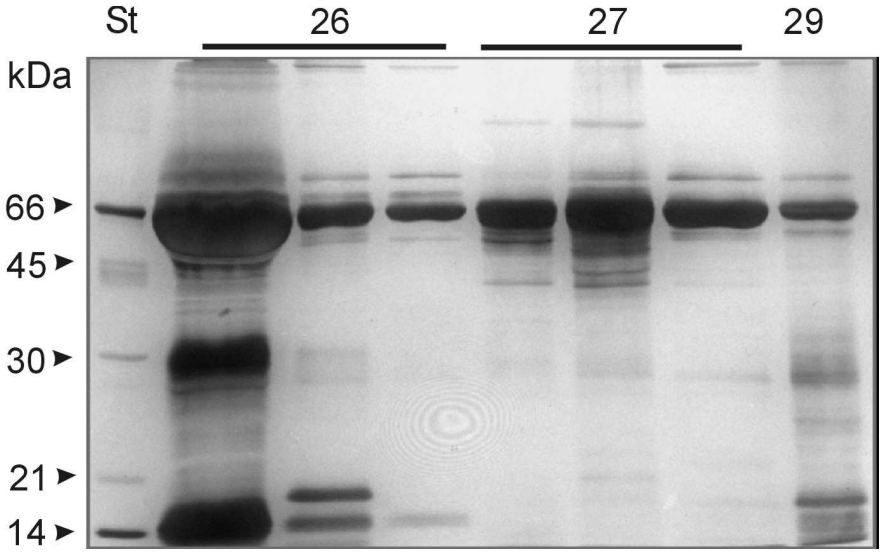

Figure 8: SDS-PAGE of urine samples from 3 diseased dogs. Vertical lanes from left to right contain the standard, dog 26 (days 0 - 2), dog 27 (days 0 – 2) and dog 29 (day 0).

In figure 9 the urine samples from dogs 36 (entire female), 37 (entire male) and 38 (entire male) from days 0 and 2 are depicted and clearly show the decreasing trend in proteinuria over the 3-day test period. Based on the electrophoresis, dog 36 had mixed proteinuria on day 0 (UPC 3.22) which had normalized by day 2 to physiological proteinuria (UPC 0.3). A similar case is evident for dog 37 who exhibited clear tubular proteinuria (UPC 1.24 and URBPC 145 µg/g), which had normalized to a physiological pattern by day 2 (UPC 0.32). Dog 38 had mixed proteinuria (UPC 1.99) and the wide, distinct band around 66 kDa corresponds to the ELISA measurement of 187 mg/l albumin and UAC 317 mg/g. The SDS-PAGE of urine from dog 39 also depicts mixed proteinuria, with a clear band in the vicinity of albumin (albumin 195 mg/l and UAC 116 mg/g). Dogs 36, 37 and 38 were survivors, whilst dog 39 died.

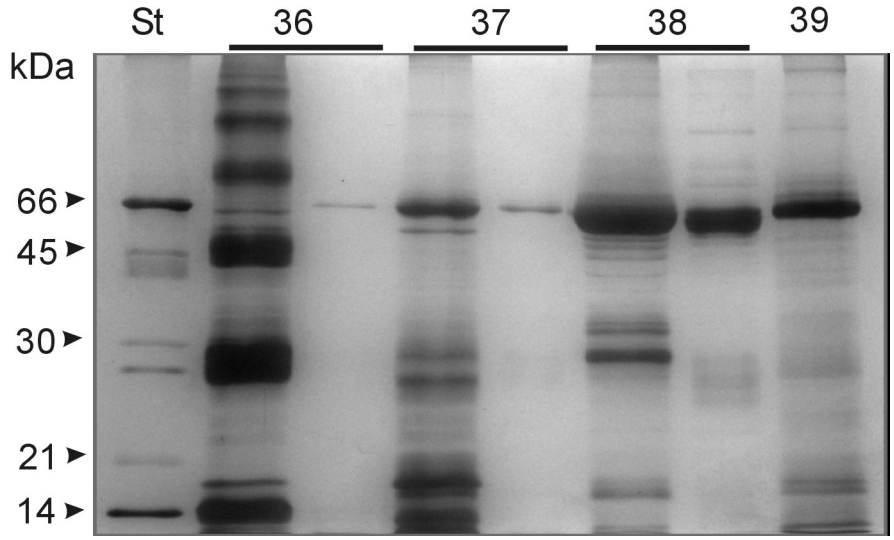

Figure 9: From left to right, standard and SDS-PAGE of urine samples from dog 36 (day 0 and 2), dog 37 (day 0 and 2), dog 38 (day 0 and 2) and dog 39 (day 0).

The differences between male and female dogs regarding LMW proteins as seen in the healthy control animals were also clearly evident in the sick animals. Although no significant difference existed in the actual number of LMW bands seen in each gender, the intensity of particular bands did appear to be gender specific. Eighteen of the 19 diseased male dogs had the pattern of one band each in the areas around 15 kDa and 30 kDa. The intensity of these bands varied greatly from dog to dog and whether the dog was neutered or not, appeared to have no influence on band intensity. Fourteen of the 19 diseased male dogs (74%) also had a band around 45-59 kDa. Although many diseased female dogs also had bands in these ranges, they quite clearly did not have the great intensity as seen in some male dogs. The protein pattern from the urine of dog 10, an entire male, as depicted in figure

10 clearly shows bands of great intensity around 15 and 30 kDa. Although bands in these molecular weight ranges are evident in the urine of dog 11, a neutered male and dog 12, a female, they are quite clearly of less intensity than seen in the urine from the entire male dog.

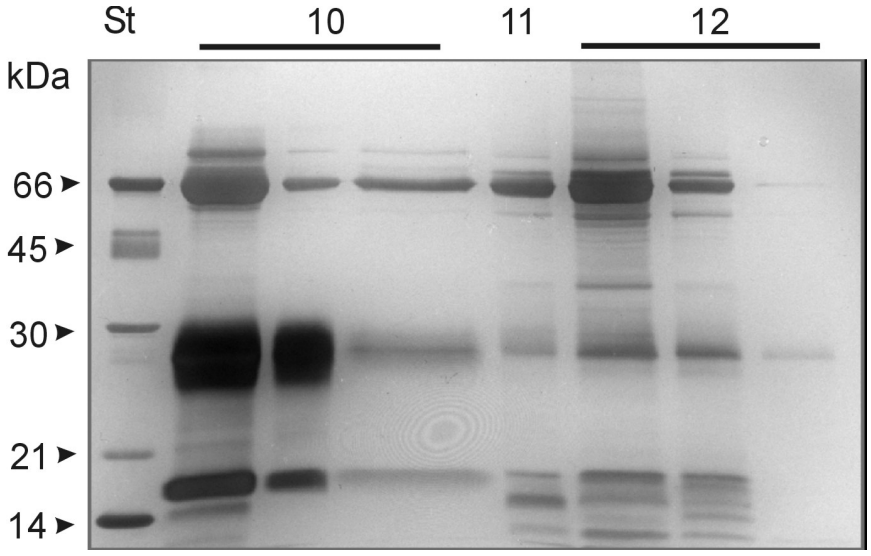

Figure 10: SDS-PAGE showing the varying patterns obtained from castrated and intact male dogs and females. Dog 10 was a sexually intact male (days 0 – 2), dog 11, a castrated male (day 0) and dog 12 was female (days 0 - 2).

4.5 Parameters for the evaluation of renal function

On day 0, the median urea concentration in serum of the diseased dogs was 5.05 mmol/l (range 2.0-53.3 mmol/l) and for healthy dogs the median was 6.3 mmol/l (range 2.8-8.3 mmol/l). No significant difference existed. Based on the in-house laboratory reference range for serum urea (3.5-10 mmol/l) used by the Clinic for Small Animals, FU Berlin, the median value for the diseased dogs was within normal range. Seven diseased dogs exceeded the upper limit on day 0 and 2 dogs developed increases in serum urea during the test period. All healthy dogs had serum urea levels within the reference range.

On day 0, the median serum creatinine level for the diseased dogs was 88.2 µmol/l (range 18-921 µmol/l), whilst the healthy dogs had a median of 80.5 µmol/l (range 56-109 µmol/l). No significant difference was evident. As was the case with serum urea, creatinine values for all healthy control dogs were within the reference range of 53-106 µmol/l for dogs < 20 kg and 53-124 µmol/l, dogs > 20 kg (Clinic for Small Animals, FU Berlin laboratory reference values). Although the total range for the diseased animals was much greater, the median was also within the reference range. Nine dogs recorded serum creatinine levels above 124 µmol/l (dogs >20 kg) at various times throughout the study period. Selected parameters can be evaluated in order to gauge the significance of pre-renal, renal and post-renal factors on

the occurrence of azotaemia (table 14).

Table 14: Selected parameters for the evaluation of renal function for the 9 diseased dogs displaying creatinine levels consistent with azotaemia.

Dog	Urea (mmol/l)			Crea (µmol/l)			UPC			K+ (mmol/l)			PCV %			USG		
	day 0	day 1	day 2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
1	53.3 *	–	–	921	–	–	0.8	–	–	3.7	–	–	59.5	–	–	1010	–	–
3	23	17	13 *	288	264	186	0.61	–	3.83	5.56	3.9	3.8	56.6	42.3	36.1	1028	–	1018
4	8.5	7	6	141	132	131	3.46	1.57	1.29	3.88	4.08	3.67	40.9	32.7	33.7	1034	1052	1045
8	8.4	– *	–	293	–	–	0.19	–	–	3.3	–	–	42.4	–	–	1038	–	–
11	– *	–	–	179	–	–	0.16	–	–	3.52	–	–	50.8	–	–	1034	–	–
14	4.8	10	7	89	134	163	0.65	0.2	0.08	4.54	3.89	3.7	41.3	31.6	32.3	1002	1006	1012
15	6.3	16	4	87	230	46	0.26	0.39	0.53	4.2	4.87	3.08	57.1	41.1	32.1	1036	1020	1012
22	35	31 *	–	305	330	–	0.5	0.91	–	3.5	5.6	–	55.3	38.6	–	1030	1022	–
38	9.4	–	–	151	–	–	1.99	–	–	2.8	–	–	15.5	–	–	1050	–	–

* denotes day of death. Abbreviations: PCV - packed cell volume, USG - urine specific gravity

In the current study the first urine sample was not always taken before fluid replacement therapy was initiated, making a definitive differentiation, based on the USG, between pre-renal and renal azotaemia difficult. However on close examination of the patient data in Table 14, it is possible to speculate that patients 4, 8, 11, 15, 22 and 38 had pre-renal azotaemia. This is based on the apparent adequate concentrating ability of the kidneys as indicated by USG measurements from day 0 that were greater than 1.030. Patient number 3 was diagnosed with a ruptured bladder secondary to a peri-anal hernia. In this case a post-renal factor contributed to the high levels of nitrogen-containing compounds in the blood. For dog 1, the extremely high level of azotaemia and the apparent haemoconcentration on day 0 paired with the isosthenuria as indicated by a USG of 1010 are highly suggestive of acute renal failure. Based on other blood results, this renal failure was highly likely due to MODS rather than being the underlying primary disease. Dog 14 had normal blood creatinine on day 0 and exhibited creatinine increases over days 1 and 2. On day 0 and 1 this dog was hyposthenuric, whilst urine from day 2 showed isosthenuria. In combination with the fluid replacement therapy, these results alone are difficult to interpret and further measurements would be necessary to deem whether renal insufficiency was present.

Eleven of the dogs in this study were treated with immuno-suppressive doses of cortisone after diagnosis of their underlying disease, IMHA. In regards to the UPC, RBP, URBPC, urinary albumin and UAC levels as well as serum urea and creatinine, no difference of any significance was observed between these dogs and the others in the diseased group.

5 Discussion

In recent years, numerous studies in human medical fields have aimed at improving understanding of the nature and development of SIRS as well as facilitating accelerated identification of patients at risk and providing efficient methods for prevention and treatment of this condition (Spapen et al., 2005; Lipsett, 2006; Sprung et al., 2006). In order to do this, emphasis has been placed on the search for biomarkers that could indicate not only the occurrence of SIRS, but also possibly the illness severity. In particular, the occurrence and severity of microalbuminuria as well as its possible prognostic value have been investigated to some extent in human medicine (Gopal et al., 2006). Following these examples, we examined urine samples from dogs with SIRS caused by various underlying illnesses and focused on immediate changes in the kidney's ability to excrete or reabsorb proteins. We investigated whether proteinuria was of a glomerular, tubular or mixed nature and also whether proteinuria occurrence or severity could provide prognostic information.

5.1 Establishment of reference values for RBP and the urinary RBP to urinary creatinine ratio (URBPC)

To date no reference values for canine urinary RBP have been established. It is therefore necessary to define a level above which urinary RBP is considered to be abnormal. Previous studies using various detection methods to investigate the presence of RBP in urine of dogs have provided varying results. Raila et al (2000) and Yalcin (2004), used immunoblot, a less sensitive study than ELISA to qualitatively detect the presence of RBP in urine of healthy dogs. In one study (Raila et al., 2000) RBP was detected in none of the urine samples from healthy dogs whilst in the other study (Yalcin and Cetin, 2004) 8% of the urine samples from healthy dogs indicated the presence of RBP. A recent study on 8 healthy dogs (defined as "healthy" by a GFR > 90 ml/min/m^2 body surface area and a UPC <0.5) recorded a median URBPC of 10 µg/g and a range of 7-30 µg/g (Raila et al., 2006) using ELISA. These values are comparable to the healthy dogs in this study (median URBPC 18 µg/g, range 9.6 – 33.8 µg/g). In a study of 118 human males, chosen randomly from a designated population, an RBP ELISA showed a mean urinary RBP content of 64 µg/l (range 10 -540 µg/l) (Topping et al., 1986), whilst in another study of a population of humans with normal renal function the authors report a median RBP excretion of 32 µg/l (range 2 -146 µg/l) (Berg et al., 1991).

In the current study, an ELISA was also used to determine urinary RBP excretion levels, resulting in a median urinary RBP excretion of 36.5 µg/l (range 27-67 µg/l) and median

URBPC of 18 µg/g (range 9.6-33.8 µg/g) for the healthy control dogs. In order to establish reference values for canine urinary RBP excretion, the following method as described previously by Bernard et al (1987) was used. The upper limits were defined as the mean value from the healthy controls plus 2 times the standard deviation (mean + 2SD). Therefore in the current study, urinary RBP excretion was considered above normal when exceeding 60.2 µg/l and URBPC was considered above normal when it exceeds 33.3 µg/g.

Based on these calculated ranges for normal urinary RBP excretion, one healthy dog marginally exceeded (33.8 µg/g) the upper limit for URBPC. In regards to the diseased dogs on day 0 however, 36 dogs (92%) had an URBPC in excess of 33.3 µg/g and 37 dogs (95%) had a urinary RBP excretion exceeding 60.2 µg/l. Eleven of the 13 non-survivors (85%) had a URBPC exceeding 33.3 µg/g and 25 of the 26 survivors (96%) also exceeded this level for URBPC on day 0.

5.2 The effect of SIRS on the renal passage of proteins

5.2.1 Occurrence and duration of proteinuria

This study showed that proteinuria is a common biochemical abnormality in dogs with SIRS. In this study, 3 parameters namely total protein, albumin and RBP excretion were examined to quantify and qualify the proteinuria. On day 0 of the examination period, 27 of the diseased dogs (69%) had proteinuria, as indicated by a UPC greater than 0.5, whilst none of the healthy control dogs had a UPC above 0.5. The difference in UPC levels between the sick dogs on day 0 and the healthy controls was highly significant. Urinary albumin and RBP excretion were also both significantly higher in the diseased dogs compared with the healthy counterparts.

Regarding the duration of the proteinuria, studies on human SIRS patients report that concurrent to the subsiding immune response, the level of proteinuria should also decline within 48 hours at the most after ICU admittance (Fleck et al., 1985; Abid et al., 2001; Gosling et al., 2003). In this study serial measurements throughout the 48-hour examination period demonstrated a declining trend for excretion of total protein and albumin (Wilcoxon $p < 0.05$) supporting the reported transience of proteinuria. No significant decline in RBP excretion could be established however, suggesting that the pathological mechanisms behind increased urinary RBP excretion may take longer than 48 hours to subside.

5.2.2 Origin of the proteinuria

As previously stated, the results from day 0 showed that 69% (n=27) of the diseased dogs had a UPC level higher than the accepted physiological level, 64% (n=25) had UAC levels consistent with microalbuminuria and 92% (n=36) of the diseased dogs had a URBPC above the determined reference value of 33.3 µg/g. In order to truly evaluate however whether SIRS leads to glomerular, tubular or mixed proteinuria, it is necessary to analyse all three parameters together as shown in table 15.

Table 15: Analysis of the simultaneous occurrence of increases in urinary proteins on day 0, displayed as the absolute number and percentage of the diseased dogs (n=39) exhibiting each group of characteristics.

1	2	3	4	5	6
UPC ↔	UPC ↑	UPC ↔	UPC ↔	UPC ↑	UPC ↑
UAC ↔	UAC ↑	UAC ↑	UAC ↔	UAC ↔	UAC ↑
URBPC ↔	URBPC ↑	URBPC ↑	URBPC ↑	URBPC ↑	URBPC ↔
2 dogs (5%)	21 dogs (54%)	3 dogs (8%)	7 dogs (18%)	5 dogs (13%)	1 dog (2%)

Accepted normal ranges: UPC < 0.5, UAC < 30 mg/g, URBPC < 33.3 µg/g.

↑ = above the accepted upper norm, ↔ = within accepted normal range

Only 2 dogs (5%) had all 3 values in the normal range (group 1) whilst the narrow majority of diseased dogs (54%) had higher than normal values for all three parameters (group 2). Through addition of the patient numbers in groups 2, 3 and 6 it can be seen that 25 dogs (64%) had increased albumin, regardless of the other parameter and through adding patient numbers in groups 2-5 it is evident that the vast majority of dogs (36 dogs, 92%) had increased URBPC regardless of whether their UPC or albumin levels were affected. These results point to the conclusion that (1) SIRS leads often to impairment of both the glomerulus and the tubules, but (2) in almost all cases in this study, an element of tubule malfunction existed.

It is generally agreed that one of the earliest consequences of inflammatory response is increased capillary permeability and capillary leak due to the effect of inflammatory cells, pro-inflammatory cytokines and reactive oxygen species on endothelial cells (Abid et al., 2001; Spapen et al., 2005; Smith et al., 2006). Plasma levels of pro-inflammatory cytokines and NO in humans are believed to be at their peak on day 1 of sepsis (Groeneveld et al., 1997). A Japanese study with experimentally induced local inflammation in dogs, detected increased levels of serum IL-6 within 2 hours of infection (Yamashita et al., 1994) and a recent German study on sepsis in dogs reported a correlation between high levels of IL-6 on the day of

admission and severity of illness (Rau et al., 2007). Furthermore, a recent study investigating the levels of the inflammatory cytokines IL-6, IL-8 and the soluble endothelial-linked adhesion molecule 1 in human sepsis patients describes a distinct correlation between the amounts of these circulating substances and the level of endothelial damage (Hein et al., 2005). The suggestion by some authors therefore, that rapidly occurring microalbuminuria could be evidence of systemic capillary leak (Gosling et al., 2003; Spapen et al., 2005), appears supported in the current study through the high levels of protein excretion recorded for the diseased dogs at their initial presentation.

The immune processes occurring during SIRS may lead to proteinuria, not only through altering capillary permeability, but also due to other effects on the integrity of the glomerular filtration barrier. As reported by Grauer (2005), glomerular damage can be caused by deposition of circulating antigen-antibody complexes in the glomerulum and the formation of immune complexes within the glomerular capillary walls. As previously mentioned, an important factor in minimizing the permeation of anionic plasma proteins is the net anionic charge of the glomerular filtration barrier. The deposition of circulating cytokines and immune complexes during SIRS could damage the perm-selectivity of the glomerular filtration barrier by disturbing these charge characteristics. Interactions between circulating molecules and the GBM are charge dependent, which influences the deposition of antibodies, antigens and immune complexes in the glomerular capillary wall (Madaio et al., 1984; Batsford et al., 1991). As shown experimentally and in connection with anti-GBM nephritis, cationic antibodies bind to antigenic sites in the glomerular capillary wall with an affinity that is four times greater than anionic antibodies (Madaio et al., 1984). Even the presence of positively charged regions on molecules with an otherwise net anionic charge, can greatly increase their affinity for the GBM (Batsford et al., 1991). The rate of glomerular deposition of antibodies is also reportedly related to their concentration in blood (Madaio et al., 1984). Due to the large amount of circulating immune cells, complement products and inflammatory cytokines during SIRS, it is quite possible that they interact with the glomerular filtration barrier in such a way. The deposition of immune complexes could cause disturbances in the charge interactions between the transmembrane proteins of the podocyte foot processes, leading to disfigurement of the epithelial slit diaphragm, increased pore sizes and therefore, increased passage of moderate to high molecular weight proteins.

Based on this knowledge, it appears probable that the proteinuria recorded within 24 hours of the SIRS diagnosis for the dogs in this study, could be due to SIRS-induced increases in capillary permeability. Impairment of the GBM filtering properties through the deposition of immune complexes could also have contributed to the proteinuria, however this was not directly investigated and the hypothesis should be proven in further studies. Given also the

fact that increased glomerular filtration can lead to an overload of the tubular reabsorption capacity and the emergence of mixed proteinuria (Biewenga and Gruys, 1986; Waller et al., 1989), it is feasible that glomerular hyperfiltration was at least to some extent, a mechanism behind the development of tubular proteinuria.

Ten dogs had URBPC > 33.3 µg/g without any noticeable increase in the UPC (figure 15, columns 3 and 4) which may be characteristic for the lower levels of proteinuria known to occur when the damage is primarily tubular (Bernard et al., 1987; Waller et al., 1989; Lulich and Osborne, 1990). RBP, a marker for tubular malfunction was present not only in the dogs with microalbuminuria but also in 12 dogs with normal albumin excretion (figure 15, columns 4 and 5). It seems therefore, that the presence of LMW proteins in urine of dogs with SIRS is not only due to saturation of tubular reabsorption mechanisms with HMW proteins and competition for binding sites, but could also be due to direct tubular damage induced by SIRS. One author implies this possibility in a study of the effects of TGF-β1 and TNF-α on tissue cultures of rat proximal tubular epithelial cells (Kanalas and Hopfer, 1997). Exposure of tubular epithelial cells to TNF-α caused a decrease in the expression of megalin, which is the most important receptor for re-uptake of LMW proteins in the renal tubules (Christensen and Birn, 2001; Christensen, 2002). The high level of urinary RBP excretion in the diseased population of this study suggests that SIRS may induce at least a transitional impairment of this function of megalin. In addition to this, TGF-β1 appeared to be involved in decreasing plasminogen activation and therefore fibrinolysis, which is of consequence for the breakdown of microthrombi and ensuing tissue hypoxia. Even when RBP levels are greatly increased, their fraction of the total urinary protein is so small that changes in RBP excretion may occur before any other abnormalities, either clinical or chemical, are noticed. Although the possible effect of inflammatory cytokines on expression of protein receptors in tubular epithelial cells has received little attention in the literature, this hypothesis may explain the increased presence of megalin ligands, such as RBP, in urine during times of high serum levels of TNF-α (Kanalas and Hopfer, 1997).

Although not fully elucidated, a connection has been established between glomerular proteinuria and progressive tubular atrophy and interstitial fibrosis in both humans and dogs. As previously mentioned direct exposure of proximal tubular cells to albumin, transferrin and IgG may lead to local expression of pro-inflammatory cytokines such as transforming-growth factor-β (TGF-β), endothelin, IL-8 and monocyte chemoattractant protein 1 (MCP-1) as well as up-regulating renal genes encoding vasoactive and pro-inflammatory cytokines (Perico et al., 2005). One of the effects of these cytokines is enhancing chemo-attraction for fibroblasts and macrophages and inducing fibroblast proliferation and the synthesis of extracellular matrix, thus leading to extra-tubular fibrosis (Berridge, 2004). In the course of these

processes a decrease in the GFR will also activate the renin/angiotensin system. Angiotensin II mediates constriction of efferent arterioles with the effect of restoring glomerular hypertension. Glomerular hypertension in itself has the side effect of increasing the drive of proteins over the glomerular filtration barrier (Grauer, 2005; Perico et al., 2005) and herewith compounding the initial problem of proteinuria. It is also hypothesized that chronic proteinuria and the consequent excessive lysosomal endocytosis induces apoptosis in tubular epithelium cells (Erkan et al., 2005). The malfunction of tubular reabsorption mechanisms in such cases was reported in one study, which investigated the tubular uptake and catabolism of LMW proteins in rats with experimentally induced nephrosis. A high level of proteinuria was related to a decreased uptake and catabolism of LMW proteins as well as an increased excretion of N-acetyl-glucosaminidase (NAG). The amount of excreted NAG, an enzyme located in lysosomes of the proximal tubular cells, is used to measure tubular cell injury. These authors associated chronic proteinuria with damage to tubular epithelial cells, which could not be restored quickly, even after therapy had ameliorated the original glomerular source of the proteinuria (Haas et al., 2003). The situation of chronic exposure to proteinuria with ensuing fibrosis does represent a rather different aetiology to many of the dogs in the current study, where acute but transient proteinuria occurred in connection with inflammatory response to acute critical illness. However, in the occurrence of both acute and chronic proteinuria, inflammatory cytokines are involved, renal hypotension occurs and mechanisms are initiated to maintain the GFR. It remains therefore speculative, to which extent acute glomerular proteinuria could have a detrimental effect on tubular function, not just through overloading tubular reabsorption capacity, but also possibly through the negative effects of acute exposure of the tubular epithelium to an over-abundance of filtered proteins. In addition to this, some of the underlying illnesses discussed in this study, such as prostatic abscess, pyometra and lymphosarcoma, which are known to be associated with high levels of circulating immune complexes (Nelson and Couto, 2003) could well have been present for some time before onset of the acute critical condition. This issue has also been noted by other authors in similar studies (Rau et al., 2007).

5.3 Pre-renal, post-renal and renal factors influencing proteinuria

When examining the presence of protein in urine, the current state of kidney function must be taken into consideration as proteinuria is often an accompanying characteristic of renal disease (Vaden et al., 1997; Jacob et al., 2005; Lees et al., 2005). As there was no knowledge of each dog's renal function prior to the onset of the critical illness, it cannot be fully ascertained whether the onset of proteinuria was entirely triggered by the effects of

SIRS or whether the effects of SIRS exacerbated pre-existing sub-clinical renal impairment. However, thirty of the diseased dogs had levels of serum creatinine within the accepted reference range (53 – 106 µmol/l for dogs < 20 kg and 53 – 124 µmol/l, dogs > 20 kg, Clinic for Small Animals, FU Berlin laboratory reference values) so that pre-existing renal impairment is unlikely. Nine dogs had serum creatinine levels above this reference maximum and were designated to have pre-renal (n=6), post-renal (n=1) and renal azotaemia (n=1). For one dog the recorded values indicated a borderline renal insufficiency, which would require repeated investigation beyond the 3-day examination period in order to produce a conclusive diagnosis.

In human medical literature, acute renal failure with serum creatinine >265 µmol/l and proteinuria is described for patients with MODS and SIRS (Johnson, 2004), however there are few reports concerning this complication in critically ill dogs. It has been reported in human medical studies that critically ill patients, requiring renal replacement therapy due to acute renal failure, had significantly higher mortality rates than those without renal failure but similar illness severity scores (Metnitz et al., 2002). Another recent study investigated the influence of intact renal function on the renal removal of pro-inflammatory cytokines (IL-1, IL-6, TNF-α) from plasma in the first 24 hours of sepsis diagnosis. The freely filtered pro-inflammatory cytokines are believed to be at peak concentration at this time and are present in urine because their ultra-filtrate load saturates tubular reabsorption capacity (Graziani et al., 2006). The authors compared cytokine levels in plasma and urine in patients with non-oliguric acute renal failure (NOARF) and oliguric acute renal failure (OARF). They report that intact renal function was vital in modulating plasma concentrations of pro-inflammatory cytokines, as significantly higher cytokine plasma levels were found in OARF patients. Prognosis was less positive for patients without sufficient diuresis (OARF patients), leading the authors to surmise that plasma pro-inflammatory cytokine levels were possibly inversely related to survival.

Fluid replacement therapy has reportedly a positive influence on prognosis for dogs following surgery for gastric dilatation-volvulus as it is believed to reduce the risk of developing hypotension and possibly ARF (Beck et al., 2006). All dogs in this study did receive fluid replacement during the course of the treatment in the clinic, however the relationship between adequate fluid replacement in dogs with SIRS and its prognostic importance, especially in regards to the development of ARF warrants further examination.

In regards to pre-renal factors affecting proteinuria, it has been reported that hypertension and an increase in GFR occur during long-term immunosuppressive glucocorticoid administration, as it does with untreated hyperadrenocorticism (Hurley and Vaden, 1998). This has shown to be associated with the development of glomerulosclerosis in laboratory

animals, humans and dogs (Ortega et al., 1996). The 11 dogs which were diagnosed with IMHA were presented for the first time for this problem and none of these dogs had previously received long term glucocorticoid therapy. Despite the application of immunosuppressive doses of prednisolone at the beginning of their treatment period, these 11 dogs did not differ statistically from the other candidates.

5.4 Further classification of the proteinuria origin through SDS-PAGE

The electrophoresis patterns of the healthy dogs in this study appear representative of normal urine samples, as similar results have been obtained by other authors (Zaragoza et al., 2003; Zaragoza et al., 2004; Zini et al., 2004). Zaragoza et al. (2003) reported 4 different bands with the prevailing presence of a faint band in the area of albumin, a band around 10-20 kDa occurring in 50% of the subjects and some urine samples containing bands at 20-30 and 30-40 kDa. In the current study, where 3 different bands were observed, albumin was also the most predominant, albeit faintly stained band. 43% of the dogs had faint bands in the 10-20 kDa range, whilst 21% of dogs showed a band at 20-30 kDa. It has been reported that dogs with no impairment in kidney function regularly show a band at 69 kDa which is considered physiologic providing the animal's UPC is below 0.5 (Zini et al., 2004) This was the case for all healthy control dogs in the current study. A band around 100-110 kDa, believed to be Tamm-Horsfall protein (THP), which is produced in the epithelial cells of the thick ascending limb of the Henle loop and the distal convoluted tubule cells and is excreted into the tubule lumen as a physiological component of canine urine (Schweigert et al., 2002; Bachmann et al., 2005). THP is often present in electrophoresis of urine from healthy dogs (Yalcin and Cetin, 2004; Zaragoza et al., 2004). Although this protein was absent in urine samples from healthy controls in this study, this need not necessarily imply a malfunction of the distal tubules, but might be ascribed to a faint staining of THP by silver nitrate solution (Raila, personal communication). Uniform for all analysis of healthy urine samples though, was the lower number of bands altogether, the absence of bands larger than 110 kDa and also lower amounts of LMW proteins than observed for diseased dogs.

In this study, eleven individual bands were identified in urine from the diseased dogs, which is similar to the findings of Zaragoza et al (2004) in dogs with pyometra, where nine bands were identified. The high frequency of bands in the regions 60-70 kDa (90%), around 100 kDa (70%) and 20-30 kDa (57%) was also very similar to findings from both Zaragoza et al (2003) and Yalcin (2004) for dogs with a UPC>0.2 and a suspected nephropathy. Yalcin (2004) used Western Blot to confirm the identity of a band around 21 kDa as RBP and reports its presence in all urines of dogs with proteinuria. A distinct band representing RBP is

difficult to detect when using only SDS-PAGE due to the proportionally small amount of RBP present, even when the amount is above normal. In the case of the diseased dogs in this study therefore, an SDS-PAGE pattern of LMW proteins allowed the suspicion that RBP was present but the actual level of RBP excretion was confirmed through the urinalysis. The presence of tubular lesions was further emphasized in SDS-PAGE through the high numbers of diseased dogs with bands in the ranges 10-20 kDa (77%) and 20-30 kDa (57%) as well as the combined percentages of dogs with tubular (28%) and mixed (46%) proteinuria, as shown in Table 15. Zaragoza et al. (2004) also reported a high prevalence of LMW bands in dogs presenting with pyometra, even to the point that some LMW proteins in the 30-60 kDa range were found only in urine of these patients but not at all in healthy controls. A similar distribution was found to occur in the current study, where only 45% of bands from healthy controls were LMW as opposed to 58% of bands in the diseased dogs.

Whilst the parameter UPC allows an initial diagnosis of increased protein excretion, ELISA is used for the quantitative determination of selected proteins and SDS-PAGE provides a qualitative evaluation, it is through the combination of all this information that an assessment can best be made as to whether the proteinuria was primarily of glomerular, tubular or mixed origin. In Table 16, the visually obtained analysis of SDS-PAGE is compared with results from the urinalysis. The majority of the diagnosis (glomerular, tubular or mixed proteinuria) derived by electrophoresis were supported through corresponding UPC, albumin and RBP levels. The median UPC for SDS-PAGE specimens likely to have proteinuria of tubular origin was 0.69 compared to 1.39 for those where proteinuria was deemed mixed or glomerular. This corresponds to the established tendency that tubular proteinuria is usually milder than glomerular proteinuria (Bernard et al., 1987; Waller et al., 1989; Lulich and Osborne, 1990). Although SDS-PAGE has been reported as highly sensitive in recognizing glomerular or tubular proteinuria, it is difficult to distinguish a difference in staining intensity between normal albumin levels and very mild microalbuminuria (Zini et al., 2004). The discrepancy between the SDS-PAGE interpretation and urinalysis results for dogs 7, 11, 18, 19, 27, 31 and 37 could possibly be thus explained. The possible fragmentation of albumin into LMW breakdown products has also been reported (Yalcin and Cetin, 2004), which could be the reason why the glomerular participation in dogs 32, 33 and 34 was not recognized and might also account for the very intense staining of LMW proteins which was evident. In some cases, such as dogs 15, 16, 20 and 28 which had normal UPC and albumin levels, but high RBP levels, the milder level of a purely tubular proteinuria was not detected in the SDS-PAGE evaluation. These dogs were all females, which raises the question as to whether gender specific differences in LMW protein excretion also played a role in the lack of visual evidence for tubular proteinuria.

Table 16: Compilation of urinalysis results and SDS-PAGE analysis for the diseased dogs on day 0.

Dog number	Sex	UPC	Albumin mg/l	UAC	URBPC	RBP µg/l	Visual analysis of SDS-PAGE
1*	F	0,8	10	28.6	537	188	Tubular
2	M	0,37	15	13.2	62.3	71	Tubular
3*	Md	0,61	15	17	96.6	85	Tubular
4	M	3,46	260	263	156	154	Mixed
5	F	1,08	32	97	597	197	Tubular
6	F	4,98	403	527	54.5	42	Glomerular non-sel.
7	M	2,68	21,7	75.6	345	99	Tubular
8*	F	0,19	25	5.6	29.3	129	Physiological
9	M	3,47	233	358	149	97	Mixed
10	M	0,81	43,5	48.9	143	128	Mixed
11*	Md	0,16	31	11.3	44.4	122	Mixed
12	F	0,65	181	80.4	62.2	140	Mixed
13	F	0,09	13	3.1	22.6	95	Physiological
14	F	0,65	14	104	526	71	Tubular
15*	F	0,26	18	9.9	85.6	155	Physiological
16	F	0,16	2,4	4.9	156	76	Physiological
17*	F	0,59	72	53.3	78.5	106	Mixed
18	F	0,43	22	72.1	325	99	Physiological
19	F	0,65	33,6	17.1	74.1	146	Mixed
20	F	0,25	3	3.7	169	138	Physiological
21	M	2,41	80	32.5	83.3	205	Mixed
22*	Md	0,49	44	29.3	70.7	106	Mixed
23*	M	0,63	11	10.4	98.1	104	Tubular
24*	M	2,62	491	94.4	31.3	163	Mixed
25	F	2,13	180	295	230	140	Mixed
26	Md	1,29	530	224	58.2	138	Mixed
27	F	0,6	14	15.2	110	101	Mixed
28	F	0,19	5	26.3	153	29	Physiological
29*	F	0,72	70	123	162	92	Mixed
30	Md	1,5	258	396	166	108	Glomerular non-sel.
31	F	4,8	10	15.6	234	150	Mixed
32*	M	3,82	313	225	131	182	Tubular
33	M	0,11	11	82.1	108	140	Physiological
34	F	7,94	107	1338	788	63	Tubular
35	M	0,4	4	5	101	81	Tubular
36	F	3,22	61	41.8	74.7	109	Mixed
37	M	1,24	45	43.3	145	151	Tubular
38	M	1,99	187	31.8	299	176	Mixed
39*	M	0,11	195	116	85.1	143	Mixed

* denotes non survivor, F=female, M=male, d= desexed

5.5 Gender-specific differences observed in urinary protein patterns

It has been reported that certain prostate-specific proteins of 15 and 30 kDa in size are found in the urine of entire male dogs (Teinfalt et al., 2000; Tsuchiya et al., 2005). Whether this leads to a significant difference in total urinary protein content between males and females is however debatable as some authors report that urine samples from male dogs, obtained after voluntary micturition, were higher in protein than those from females (Barsanti and Finco, 1979), whilst others report no difference between entire males, neutered males and females (Teinfalt et al., 2000). When comparing the 3 entire healthy males with the remaining healthy dogs (n=12) in the current study, a significant UPC difference ($p<0.05$) was evident, whilst a comparison between healthy males (n=9) and healthy females (n=6) showed no significant difference. In regards to the diseased dogs, no gender difference was observed for total urinary protein content although it is quite possible that the higher levels of proteinuria recorded for the diseased dogs masked any potential gender-specific differences. All healthy dogs in this study had normal UPC values so that the scope of gender-specific differences appears small. The 3 entire healthy males did indeed have the most bands in the SDS-PAGE, each with bands of varying intensities at 15, 30 and 66 kDa, although two females and one neutered male also had faint bands at 15 kDa. The 15 and 30 kDa proteins were found in SDS-PAGE in 18 of the 19 entire male diseased dogs, although a large percentage of the diseased dogs, regardless of gender, also had proteins in these molecular weight ranges. This might be due to the high incidence of tubular and mixed proteinuria and therefore the abundance of LMW proteins observed in the SDS-PAGE, so that further tests would be necessary to accurately identify the presence of gender-specific proteins. One striking difference however was evident, in that the 15 and 30 kDa bands observed in entire males as shown in figure 10 were of an extremely strong intensity not observed in the other dogs.

In their study on induced renal papillary necrosis, which included only entire male dogs, Tsuchiya et al (2005) reported finding two abnormal proteins in the urine of dogs during various stages of illness. Tissue kallikrein is a 40 kDa acidic glycoprotein present in several tissues including the kidney where it is involved in renal haemodynamics and in promoting excretory functions through the kallikrein-kinin system. The authors report that a precursor of renal kallikrein was detected as a distinct band around 30 kDa in the urine of dogs with mild renal damage and that its expression was probably increased as a compensatory measure to improve haemodynamics. They further report that Clusterin, an 80 kDa glycoprotein also known as “testosterone-repressed prostate message-2” is produced only in entire males and supposedly functions in the regulation of complement activity. Its expression is found to be up-regulated in severely injured renal tissue. In their study, Clusterin precursors were evident

in SDS-PAGE electrophoresis at 40 kDa in urine of the male dogs with renal papillary necrosis but were not found in urine from healthy dogs. In the current study, due to the high presence of bands detected around 30 kDa, an increased presence of a 30 kDa kallikrein precursor could be neither confirmed nor denied. Five entire male dogs did indeed have a very distinct band around 40 kDa which could possibly represent Clusterin, a supposition strengthened by the fact that none of the desexed male dogs exhibited this band. However, as once again 4 female dogs had very distinct bands in this area, subsequent tests would be necessary in order to properly confirm the protein's identity and its validity as a marker for renal papillary damage.

5.6 Prognostic value of proteinuria

Despite the significantly higher total urinary protein, albumin and RBP excretion exhibited by dogs with SIRS compared to healthy dogs, no significant difference was recorded when comparing survivors and non-survivors for either day 0 or day 2. The initial severity of proteinuria, albuminuria and urinary RBP excretion in diseased dogs as well as the levels 48 hours later did therefore not allow any prognosis of mortality. Although it appears that SIRS influences renal protein filtration and reabsorption in dogs, there seems to be no correlation between either the occurrence of acute proteinuria and mortality nor proteinuria severity and mortality. In human medical studies, contradicting outcomes have been obtained regarding MA and mortality prediction. These range from no correlation at all (De Gaudio et al., 1999) to a direct correlation between MA, illness severity and outcome (MacKinnon et al., 2000). Whether illness severity is mirrored by MA severity in dogs remains to be seen. Scoring systems, such as used in human medicine are not yet established in veterinary medicine, making it difficult to accurately compare individual animals or study populations in different trials. Investigations into the relationship between time course, cytokine levels and endothelial damage in ICU patients who developed septic shock (Hein et al., 2005) have shown that serum levels of Soluble Endothelial-linked Adhesion Molecule 1 (SELAM-1) in non-survivors tended to increase further during the first 48 hours in the ICU whereas survivors were shown to have decreasing serum levels of this cytokine. Such investigations support the idea that increasing or subsiding levels of MA have higher implications for prognosis rather than the initial occurrence of MA (Abid et al., 2001). Ten of the 13 non-survivors in the current study provided only one urine sample as their demise occurred before further urine collection and analysis could be carried out. However, the three non-survivors for whom serial measurements were possible (dogs 3, 15 and 22) showed increases in all 3 parameters (UPC, URBPC and UAC) from days 0 to 2. Although only one

of the survivors (dog 30) exhibited comparable increases in all three proteinuria parameters, to satisfactorily investigate the relationship between increasing or decreasing proteinuria and the survival chances of dogs with SIRS, a much larger patient group with an extended investigation period would be needed.

5.7 Conclusions

In the current study, dogs diagnosed as having SIRS demonstrated a significantly higher excretion of urinary proteins in comparison to healthy dogs. Within the diseased group, UPC, UAC and URBPC values at presentation were above normal for 27 (69%), 25 (64%) and 36 (92%) dogs respectively. In regards to albumin, the marker protein for glomerular proteinuria, it appeared that glomerular hyperfiltration could have played a role in the increased protein excretion. The increased presence of RBP in urine, regardless of whether or not UPC or UAC were increased, indicated also that an element of tubular malfunction was present in almost all diseased dogs.

The diseased and healthy groups at clinical admission further differed in the number and classification of protein bands observed in SDS-PAGE. When using visual evaluation of SDS-PAGE to classify proteinuric patterns, all healthy dogs were interpreted to have physiological patterns. In the diseased group, 8 dogs (21%) exhibited a physiological pattern, 2 dogs (5%) showed non-selective glomerular patterns, in 11 (28%) dogs only proteins of tubular origin were visualised and 18 dogs (46%) demonstrated the involvement of both glomerular and tubular mechanisms leading to proteinuria. This further emphasized the influence of tubular mechanisms on the occurrence and characteristics of the proteinuria in dogs with SIRS.

Although no relationship was apparent between the occurrence and severity of proteinuria and the mortality rate, it would be of interest in future studies to more closely investigate normalisation of protein excretion and prognosis. Whether a decrease in urinary protein levels within 48 hours of presentation at a veterinary clinic is associated with a higher chance of a positive outcome, or whether failure of urinary protein excretion levels to decrease within this time frame is a factor associated with a negative prognosis, warrants further investigation with a larger patient collective.

Until now, reference values for the urinary content of RBP have not been available for dogs. The level of RBP in canine urine and the RBP to creatinine ratio (URBPC) recorded for the healthy and diseased dogs in the current study could be used to help define reference values in future studies.

6 Summary

In response to severe injury or illness, inflammatory mediators initiate a systemic pro-inflammatory reaction, which is in turn attenuated or counteracted by the activation of modulating anti-inflammatory mechanisms. Severe inflammatory response syndrome (SIRS) is the state that occurs when an imbalance arises between pro- and anti-inflammatory responses. As a consequence of SIRS, endothelium cells are activated, vascular permeability is increased and vasodilatation, hypovolaemia, hypotension and tissue hypoxia occur. Increased renal filtration of plasma proteins is believed to result from these processes. The definition and diagnostic criteria for SIRS in humans, as developed in 1992, has recently been adapted by veterinarians for the recognition of SIRS in dogs.

In the current study, 39 dogs with SIRS and 15 healthy dogs were examined for the presence, quantity and quality of proteins in their urine. Urine and blood samples were obtained from the diseased dogs on admission to a veterinary hospital as well as 24 (n=16) and 48 (n=24) hours thereafter, whenever possible. Urinalysis was conducted for total protein content and creatinine in order to calculate the urinary protein to creatinine ratio (UPC). Using ELISA, urinary albumin and RBP levels were also quantified and the ratios, albumin to urinary creatinine (UAC) and RBP to urinary creatinine (URBPC) were calculated. SDS-PAGE was conducted for each dog to further identify the proteinuric pattern as indicative of low molecular weight (LMW) or middle molecular / high molecular weight (MMW/HMW) proteinuria. Furthermore, reference ranges for the level of urinary RBP and the URBPC were defined which could be used to help further establish reference values for future studies.

This study showed that proteinuria is a common occurrence in dogs identified as having SIRS. Diseased dogs had significantly much higher levels for excretion of total urinary protein, UPC, albumin and RBP compared to the healthy dogs. 27 diseased dogs (69%) had a UPC > 0.5, 25 dogs (64%) had a UAC > 30 mg/g indicating microalbuminuria to overt proteinuria, whilst 36 dogs (92%) had a URBPC level above the defined upper limit of 33.3 µg/g. The incidence of abnormally high RBP in urine, regardless of whether or not UPC or albumin values were increased, indicated that an element of tubular malfunction was present in almost all diseased dogs. Whilst the UPC and UAC levels were significantly higher at admission than 48 hours later, this was not found for RBP, indicating that although glomerular proteinuria begins to subside within this time, tubular impairment may have a longer duration.

The protein patterns obtained through visual inspection of SDS-PAGE showed great difference between the healthy and diseased dogs. The dogs with SIRS had a total of 11 different bands. Although a band in the range 60-70 kDa dominated, occurring in 36 dogs (92%), 58% of the total counted bands were in the LMW range whilst 42% were MMW/HMW. In the SDS-PAGE from the healthy dogs, 3 different bands were visible and only 45% of total counted bands were LMW. Through visual evaluation of the SDS-PAGE, all healthy dogs were deemed to have a physiological pattern. Of the diseased dogs, 11 (28%) exhibited an electrophoresis pattern indicative of tubular proteinuria, 18 (46%) had patterns suggesting mixed proteinuria, 2 (5%) had patterns indicating glomerular origins and 8 dogs (21%) were deemed to have physiological patterns.

Previously reported gender-specific proteinuric differences were evident between entire healthy males and the remaining healthy dogs in this study. This was seen through the significantly higher UPC and higher number and intensity of specific LMW bands in urine from the healthy males compared with the other healthy dogs. Although 18 of 19 entire diseased dogs exhibited these specific bands, the generally higher incidence of LMW bands in urine of diseased dogs made an identification of gender specific patterns difficult. The higher level of proteinuria in the diseased dogs may also have masked possible gender-specific characteristics as no difference in UPC was observed between entire males and others.

The search for differences in urinary protein excretion between survivors (n=26) and non-survivors (n=13) proved not remarkable as no significant differences were found between survivors and non-survivors, neither for proteinuria at admission nor on day 2. Although it appears that SIRS significantly influences renal protein filtration and reabsorption in dogs, correlations between the occurrence of acute proteinuria and mortality and proteinuria severity with mortality were not apparent. Of interest for future investigations is whether increasing or subsiding levels of proteinuria during the early stages of SIRS and in particular, individual trends in proteinuria of glomerular or tubular origin, could have implications for mortality prediction in dogs. Further investigation involving a larger patient collective would be valuable in the search for markers for the severity of a systemic inflammatory response and for evaluating prognosis in dogs with SIRS.

7 Zusammenfassung

Untersuchungen zur quantitativen und qualitativen Proteinausscheidung im Harn von Hunden mit „Severe Inflammatory Response Syndrome“ (SIRS)

Die Ausscheidung von Plasmaproteinen in den Urin ist sowohl von anatomischen und physiologischen Charakteristika der Nephrone, als auch von den molekularen Eigenschaften der Proteine selbst abhängig. Im Wesentlichen bestimmen folgende Faktoren das Schicksal der Proteine während der Passage durch die Glomeruli: (1) die Porengröße der dreischichtigen glomerulären Filtrationsmembran, (2) die Polarität der Membran, die eine Rolle in der strukturellen Aufrechterhaltung dieser Schichten spielt und (3) die Ladung und Molekular-gewicht der Proteine. Proteine mit einem niedrigen Molekulargewicht (LMW Proteine < 60 kDa) sind relativ frei filtrierbar, für mittelgroße Proteine (MMW 60-80kDa) ist eine Passage vor allem durch die polyanionische Oberfläche stark limitiert und hochmolekulare Proteine (HMW > 80kDa) können unter physiologischen Bedingungen die Filtrationsmembran nicht passieren. Proteine, die durch die glomeruläre Filtrationsmembran gelangen, werden fast vollständig mittels rezeptorvermittelter Endozytose in den proximalen Nierentubuli rückresorbiert. Die Membranrezeptoren Megalin und Cubilin sind hierfür verantwortlich. Der überwiegende Anteil der im Endharn physiologisch ausgeschiedenen Proteine wird im distalen Tubulusbereich synthetisiert und sezerniert. Auf renaler Ebene kommt es vor allem zu einer Proteinurie, wenn die strukturelle Integrität der glomerulären Filtrationsmembran beschädigt ist und erhöhte Mengen an MMW- und LMW-Proteinen diese passieren können und/oder die Rückresorptionsmechanismen in den Nierentubuli eingeschränkt oder überfordert sind. Zusätzlich können prä- oder postrenale Ursachen sowie eine Kombination dieser Mechanismen zu einer Proteinurie führen. Die Molekulargröße der Proteine im Urin gibt Hinweise auf die Lokalisation der Läsion oder Dysfunktion. Erhöhtes Vorkommen von z.B. Albumin (einem MMW Protein) im Endharn deutet auf Läsionen im Glomerulusbereich hin, wohingegen die Ausscheidung hoher Mengen von Retinol-Bindungsprotein (ein LMW Protein) charakteristisch für Tubulusläsionen ist.

Als Antwort des Organismus auf Traumata oder schwere Erkrankungen kommt es zur schnellen Freisetzung von inflammatorischen Zytokinen und Interleukinen in das Blut. Proinflammatorische Mediatoren wie z.B. IL-1, IL-6 und TNF-α reagieren an Oberflächenrezeptoren verschiedener Zellen wie Makrophagen, neutrophilen Granulozyten, T-Lymphozyten und Endothelzellen, die ihrerseits die immunologischen, patho-physiologischen und klinischen Zeichen einer inflammatorischen Antwort hervorrufen. Um eine stark überschießende Reaktion zu verhindern, sollte die systemische inflammatorische

Reaktion schon nach kurzer Zeit durch die Freisetzung von anti-inflammatorischen Mediatoren gebremst werden. Ist die ursprüngliche imflammatorische Reaktion zu stark, oder das Gleichgewicht zwischen pro- und antiinflammatorischen Reaktionen gestört, entwickelt sich ein Zustand, der „Systemic Inflammatory Response Syndrome“ (SIRS) genannt wird. Die genauen Pathomechanismen sind noch nicht vollständig geklärt, jedoch wird angenommen, dass bei einem SIRS inflammatorische Mediatoren und Immunzellen für die Aktivierung von Endothelzellen verantwortlich sind, und somit zu Vasodilatation, erhöhter Gefäßpermeabilität, Hypovolämie, Hypotension und Gewebshypoxie führen. Dies kann zur Schädigung der anatomischen Struktur und Einschränkung der physiologischen Funktion der Nieren führen, so dass letztendlich erhöhte Mengen von Plasmaproteinen über den Urin verloren gehen können.

In der Humanmedizin wurde eine Definition für SIRS sowie klinische Kriterien zur Erkennung von SIRS 1992 international festgelegt. Diese Kriterien wurden für die Veterinärmedizin adaptiert. Das Ziel dieser Studie war es, zu untersuchen, ob (1) SIRS bei Hunden zur Proteinurie führt, (2) ob Proteinmuster erkannt werden können, die auf eine glomeruläre oder tubuläre Dysfunktion rückschliessen lassen und (3) ob die Proteinurie als prognostischer Marker verwendet werden kann. Fünfzehn gesunde und 39 kranke Hunde gingen in die Studie ein. Diesen Hunden wurden bei Erstvorstellung Harn- und Blutproben entnommen und in der Gruppe der erkrankten Tiere wurden, wenn möglich, zusätzliche Harnproben nach 24 (n=16) bzw. 48 (n=24) Stunden entnommen. Mittels der Bradford-Analyse und der alkalischen Pikrat-Methode wurden jeweils Gesamtprotein und Kreatinin im Harn gemessen und ein Urinprotein-Urinkreatinin-Quotient (UPC) gebildet. Ein ELISA wurde zur Bestimmung der Urinproteinmengen von Albumin und Retinol-Bindungsprotein (RBP) verwendet und die Quotienten Albumin-Kreatinin (UAC) und RBP-Kreatinin (URBPC) errechnet. Die Proteinmuster wurden mittels einer SDS Polyacrylamid-Gelelektrophorese (SDS-PAGE) analysiert.

Die schon von anderen Autoren beschriebenen geschlechtsspezifischen Unterschiede in Proteinuriemustern wurde auch in dieser Studie beim Vergleich der Werte von gesunden nicht kastrierten Rüden und den übrigen gesunden Hunden deutlich. Sowohl UPC als auch die Anzahl und Intensität spezifischer LMW-Banden waren bei diesen Rüden deutlich höher. Obwohl bei 18 von 19 kranken nicht kastrierten Rüden die spezifischen Banden ebenfalls vorhanden waren, konnten geschlechtsspezifische Unterschiede aufgrund der insgesamtdeutlich erhöhten Anzahl von LMW-Banden in der Gruppe der kranken Hunde nicht identifiziert werden. Auch waren bei den UPC-Werten der nicht kastrierten Rüden und der restlichen erkrankten Hunde keine Unterschiede vorhanden und es ist anzunehmen, daß mögliche geschlechtsspezifische Charakteristika durch die stärker ausgeprägte Proteinurie

bei erkrankten Hunden verdeckt worden sind.

Die Studie konnte zeigen, dass eine Proteinurie bei Hunden mit SIRS häufig vorkommt. Alle gemessenen Harnparameter, d.h. Gesamtprotein, UPC, Albumin, UAC, RBP und URBPC waren im Median bei kranken Hunden am Tag 0 im Vergleich zu gesunden Tieren signifikant erhöht. 27 Hunde (69%) der kranken Gruppe hatten eine UPC >0.5, 25 Hunde (64%) hatten eine erhöhte Albuminausscheidung, nachweisbar durch einen UAC-Quotienten von >30mg/g, desweiteren war bei 36 Hunden (92%) die URBPC höher als 33.3 µg/g. Diese erhöhte RBP- Ausscheidung ohne gleichzeitig erhöhtes UPC, bzw. erhöhte Albuminwerte liess vermuten, dass bei fast allen Hunden der erkrankten Gruppe ein tubuläre Schädigung vorhanden war. Insgesamt waren die Werte für UPC und UAC am Tag 0 signifikant höher als am Tag 2, wobei diese Differenz für den Parameter URBPC nicht das Signifikanzniveau erreichte. Anhand dieser Ergebnisse ist zu vermuten, dass bei Hunden mit SIRS eine akute aber scheinbar transiente glomerulär bedingte Proteinurie auftritt, wohingegen die tubulären Schädigungen etwas länger anhalten. Die Proteinmuster im SDS-PAGE zeigten deutliche Unterschiede zwischen kranken und gesunden Hunden. Bei SIRS-Patienten trat eine deutlich höhere Anzahl von Banden auf (11 vs. 3), zusätzlich war eine größere Streuung des Molekulargewichtes zu erkennen. Bei erkrankten Hunden war am Tag 0 in 46% (n=18) der Gelelektrophoresen ein Muster für eine gemischte Proteinurie sichtbar und bei 28% (n=11) waren die Proteinmuster typisch für eine tubuläre Proteinurie. In nur 5% (n=2) der Elektrophoresen waren rein glomeruläre Proteinmuster vorhanden. Im Gegensatz dazu waren bei allen gesunden Hunden physiologische Proteinmuster zu erkennen.

Obwohl eine überwiegende Anzahl der Hunde mit SIRS bei der Erstuntersuchung eine Proteinurie aufwiesen, konnte kein Unterschied in den UPC-, UAC- und URBPC-Werten der überlebenden (n=26) und nicht-überlebenden (n=13) Hunde festgestellt werden. Im Gegensatz zu Berichten aus der Humanmedizin scheint das Vorkommen bzw. der Schweregrad der Mikroalbuminurie bei Hunden mit SIRS keine Prognose im Hinblick auf die Mortalität zu erlauben. Die Frage, ob eine Abnahme der Proteinausscheidung innerhalb der ersten 48 Stunden Rückschlüsse auf einen positiven Krankheitsverlauf zulässt, konnte durch diese Studie nicht geklärt werden. Weitere Untersuchungen mit größeren Patientenzahlen wären für die Suche nach einem Marker für den Schweregrad einer systemischen inflammatorischen Reaktion und zur Beurteilung der Prognose von Hunden mit SIRS notwendig.

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9 Appendix

9.1 Tables with compiled patient data and results

Table 17: Diseased dogs - patient data, clinical diagnosis and outcome

Dog	Breed	Age	Sex	Clinical diagnosis	Outcome
1	Dachshund	12	F	acute renal failure	died (0)
2	Leonberger	1	M	parvovirosis	released
3	Mixed breed	12	M d	peritonitis - peri-anal hernia, ruptured bladder	died (2)
4	Bernese mountain dog	7	M	prostatic abscess	released
5	Irish setter	6	F	haemometra	released
6	Rottweiler	13	F d	acute gastritis	released
7	Mixed breed	7	M	skin abscess	released
8	Golden retriever	9	F	splenic neoplasia	died (1)
9	Great dane	5	M	prostatic abscess	released
10	Great dane	3	M	mesenteric infarction	released
11	German shepherd	8	M d	fever of unknown origin	died (0)
12	Hovawart	9	F	peritonitis - ruptured gall bladder	released
13	Beauceron	7	F	salmonellosis	released
14	Sheltie	9	F	pyometra	released
15	Dachshund	12	F	pyometra	died (5)
16	German shepherd	10	F	pyometra	released
17	German shepherd	10	F	pyometra	died (0)
18	Cairn terrier	11	F	pyometra	released
19	Mixed breed	8	F	necrotizing tonsillitis	released
20	Golden retriever	10	F d	peritonitis - perforated bowel, neoplasia	released
21	Fox terrier	3	M	peritonitis - perforated bowel, foreign body	released
22	Doberman	11	M d	skin abscess	died (1)
23	Welsh terrier	4	M	peritonitis - perforated bowel, foreign body	died (0)
24	German shepherd	10	M	skin abscess	died (0)
25	Great dane	8	F	skin abscess	released
26	Great dane	4	M d	prostatic abscess	released
27	German shepherd	6	F	pyometra	released
28	Mixed breed	8	F	pyometra	released
29	German shepherd	10	F	IMHA	died (2)
30	Mixed breed	9	M d	IMHA	released
31	Doberman	9	F	IMHA	died (6)
32	Shih Tzu	7	M	IMHA	died (2)
33	Mixed breed	6	M	IMHA	released
34	Cocker spaniel	6	F	IMHA	released
35	Staffordshire terrier	7	M	IMHA	released
36	Mops	8	F	IMHA	released
37	Perro de presa mallorquina	1	M	IMHA	released
38	German shepherd	10	M	IMHA	released
39	Sheltie	10	M	IMHA	died (5)

F = female, M = male, d = desexed, day of death in brackets

Table 18: Diseased dogs - vital signs at presentation and selected biochemistry results

Dog	Temp. ℃	HR /min	RR /min	WBC day 0 cells/µl	Bands %	Urea 0 mmol/l	Urea 1	Urea 2	Crea 0 µmol/l	Crea 1	Crea 2
1	38.5	160	28	30.3	1	53.3			921		
2	40.4	184	52	31.1		4	2.9		117	77	
3	37.2	144	66	0.38		23	17.5	12.7	288	264	186
4	40.6	120	80	23.2	3	8.5	7.3	6.5	141	132	131
5	37.5	116	16	44.8		6.9			66		
6	39.5	140		20.8	0	2.5			91		
7	39.2	100		26.9	5	2.8	3.7	3.5	71	70	76
8	40	140		25.3	35	8.4			293	189	
9	39.9	180		11	7	4.3	3.6	1.9	92	85	85
10	38.9	120		18.9	10	5.7	4.8	3.9	105	73	72
11	40.5	160		25.1	3				179		
12	40.9	200		8.22	8	2.6	9.0	6.5	83	116	99
13	40.8	168	120	22	9	4	2.6	2.5	75	75	68
14	39.8	160	44	36.8	15	4.8	9.9	7.0	89	134	163
15	39.6	180	44	7.76	70	6.3	16.0	4.5	87	230	46
16	39.3	100		33.5	18	2	2.1	1.7	88	78	78
17	40.8	160	48	15.2		2.7			106		
18	40	144	66	5.9	17	4	8.3		75	80	
19	40.7	120	28	0.6		3.7					
20	39.4	100	80	17.2	3	4	3.0		68	55	67
21	38.7	180		11.3		5	2.3	2.7	61	37	39
22	36.5	120	24	2.4		35	31.5		305	330	
23	37.3	156	20	1.6	29	6.8			95		
24	41.3	148		11.8	1						
25	39.8	160	66	28.7	6	2.2	1.9		57	56	
26	40	180	30	29.6	17	4.4	4.1		75	65	
27	40			12							
28	38.8			17.3		3.5			70		
29	39.5	144	48	14.2		5.1	26.4	17.5		38	111
30		140		42.5		8.3			86		
31	39.7	92		38.4	3	12.5		9.4	106		11
32	36.9	104		44.7	20	22.4	16.6		82	78	
33	35.2			65.6	10	4.3			18		
34	40			12		3.8			59		
35	38.4	140	36	14.6	7	6.4			103		
36	39.9	132			8	10		3.4	62		46
37	39.3			24.7	3	6.9			71		
38	38.8	128		22.9	7	9.4			151		
39	42.2	140	72	16.5	6	19.9	18.1	22.7	88		102

Table 19: Diseased dogs urinalysis - total protein, creatinine, UPC and USG

Dog	Protein 0 mg/dl	Protein 1	Protein 2	Crea 0 mg/dl	Crea 1	Crea 2	UPC 0 mg/mg	UPC 1	UPC 2	USG 0	USG 1	USG 2
1	27.9			34.8			0.8			1010		
2	41.9	35.8	39.9	114.2	202	253.5	0.37	0.18	0.16	1048	1060	1060
3	53.5		82.4	87.5		21.5	0.61		3.83	1028		1018
4	342.1	78.8	88.7	98.9	50.2	68.9	3.46	1.57	1.29	1034	1052	1045
5	35.7	30.1	4.3	33	76	27.8	1.08	0.4	0.15	1012	1042	1014
6	381			76.5			4.98			1010		
7	76.8	35.1	11.5	28.7	90.3	80.7	2.68	0.39	0.14	1016	1020	1018
8	84.6			443.2			0.19			1038		
9	226	427.7	482.1	65	92.5	122.8	3.47	4.62	3.92	1018	1026	1028
10	71.8	17.6	23.1	88.8	56.1	84	0.81	0.31	0.27	1038	1018	1016
11	44.4			275.3			0.16			1034		
12	145.6	22	7.5	225.2	108	40.9	0.65	0.2	0.18	1040	1042	
13	36.9	10.2	8.4	419.5	144.6	138	0.09	0.07	0.06	1032	1016	1018
14	8.8	3.9	5.7	13.5	19.7	72	0.65	0.2	0.08	1002	1006	1012
15	47	13.1	3.9	181	33.3	7.3	0.26	0.39	0.53	1036	1020	1012
16	7.7	4.5	6.6	48.6	37.1	49	0.16	0.12	0.13	1031	1038	1041
17	79.4			134.8			0.59			1024		
18	13.1		36.8	30.5		74.5	0.43		0.49	1045		1037
19	127.8			196.8			0.65			1028		
20	20.7	19.8	5.7	81.7	128	52.2	0.25	0.15	0.11	1039		
21	591.7	8.4	94	245.8	24.8	46.8	2.41	0.34	0.2	1060	1010	1024
22	73.8		50.8	150.2		55.5	0.5		0.91	1030		1022
23	67			105.9			0.63			1048		
24	1360.7			520			2.62			1060		
25	130.1			61			2.13			1016		
26	306.8	42.8	28.9	237.2	76.3	74.2	1.29	0.56	0.39	1050	1018	1014
27	54.6	79.9	66	91.5	132.5	93	0.6	0.6	0.7	1029	1038	1032
28	3.6	3.6	30	19	68.7	99.1	0.19	0.05	0.03	1041	1046	1042
29	41			56.8			0.72			1016		
30	97.3		130.2	65.1		51	1.5		2.55	1049		
31	306.8			64			4.8			1044		
32	530.7			139.1			3.82			1047		
33	14.5			13.4			0.11			1010		
34	65.3		64.3	8.2		14.9	7.94		4.32	1016		1012
35	32.1		4.3	80.2		33.6	0.4		0.13	1022		
36	470.1		7.3	146		23.2	3.22		0.31	1048		1008
37	129.4		13.7	104.2		43.1	1.24		0.32	1020		
38	117.3			58.8			1.99			1050		
39	18.8			167.6			0.11			1037		

Table 20: Diseased dogs urinalysis - RBP and Albumin excretion levels.

Dog	RBP 0 µg/l	RBP 1	RBP 2	Alb 0 mg/l	Alb 1	Alb 2
1	188			10		
2	71	68	77	15	4.1	4.2
3	85		67	15		34
4	154	148	143	260	200	109
5	197	281	39	32		3
6	42			403		
7	99	77	42	21.7	41	19.9
8	129			25		
9	97	111	102	233	272	176
10	128	54	55	43.5	17	25.6
11	122			31		
12	140	104	125	181	53	2
13	95	49	44	13	7	10
14	71	35	67	14	5	3
15	155	85	38	18	9	22
16	76	43	53	2.4	1.1	2.4
17	106			72		
18	99		111	22		9
19	146			33.6		
20	138	80	48	3	7	5
21	205	145	136	80	5	1.6
22	106		86	44	19	
23	104			11		
24	163			491		
25	140			180		
26	138	118	79	530	9	27
27	101	172	218	14	18	16
28	29	37	28	5	3	3
29	92			70		
30	108		142	258		467
31	150			10		
32	182			313		
33	140			11		
34	63		70	107		109
35	81		142	4		11
36	109		102	61		30
37	151		139	45		11
38	176			187		
39	143			195		

Table 21: Diseased dogs – URBPC, UAC and SDS-PAGE interpretation

Dog	URBPC 0 µg/g	URBPC 1	URBPC 2	UAC 0 mg/g	UAC 1	UAC 2	SDS-PAGE
1	537			28.6			Tubular
2	62	34	30	13.2	2	1.7	Tubular
3	97		305	17		158	Tubular
4	156	295	208	263	398	158	Mixed
5	597	370	140	97	10.8	11	Tubular
6	55			527			Glomerular non-sel.
7	345	85	52	75.6	45.4	24.7	Tubular
8	29			5.6			Physiological
9	149	119	85	358	294	143	Mixed
10	143	96	66	48.9	30.3	30.5	Mixed
11	44			11.3			Mixed
12	62	96	306	80.4	49.1	4.9	Mixed
13	23	34	32	3.1	4.8	7.2	Physiological
14	526	178	93	104	25.4	4.2	Tubular
15	86	255	521	9.9	27	301	Physiological
16	156	116	108	4.9	3	4.9	Physiological
17	79			53.3			Mixed
18	325		149	72.1		12.1	Physiological
19	74			17.1			Mixed
20	169	63	92	3.7	5.5	9.6	Physiological
21	83	585	291	32.5	20.2	3.4	Mixed
22	71		155	29.3		34.2	Mixed
23	98			10.4			Tubular
24	31			94.4			Mixed
25	230			295			Mixed
26	58	155	106	224	11.8	36.4	Mixed
27	110	130	234	15.2	13.5	17.2	Mixed
28	153	54	28	26.3	4.4	3	Physiological
29	162			123			Mixed
30	166		278	396		916	Glomerular non-sel.
31	234			15.6			Mixed
32	131			225			Tubular
33	108			82.1			Physiological
34	768		470	1338		727	Tubular
35	101		423	5		32.7	Tubular
36	75		440	41.8		129	Mixed
37	145		323	43.3		25.5	Tubular
38	299			318			Mixed
39	85			116			Mixed

Table 22: Healthy control dogs – patient data, vital signs at presentation and selected haematology and biochemistry data

Dog	Age	Sex	Temp. ℃	HR /min	RR /min	WBC cells/µl	Urea mmol/l	Crea µmol/l
40	3	Md					8,3	100
41	3	F	38,8	132	28	6,61	6,8	88
42	3	M	38,8	132	32	5,2	5,1	71
43	6	F	38,6	96	34	8,32	4,8	74
44	7	Md	38,6	128	28	7,86	2,8	59
45	4	Fd	38,6	64		10,4	4,2	89
46	1	F	38,7	80	24	8,51	4,4	56
47	5	Md					4,6	76
48	4	Md	38,2	104	24	8,99	6,3	88
49	4	Md	38,7	100	24	5,53	6,8	104
50	5	Md	38	52	24	15,7	7,8	109
51	1	Fd	38,5	96	24	7,8	7	80
52	10	Fd				7,98	4,6	69
53	3	M	38,6	108	32	10,6	6,5	79
54	1	M	38,7		76	12,4	6,7	82

F = female, M = male, d = desexed

Table 23: Healthy control dogs urinalysis - total protein, creatinine, UPC, RBP, URBPC, albumin, UAC, USG and SDS-PAGE

Dog	Protein mg/dl	Crea mg/dl	UPC	RBP µg/l	URBPC µg/g	Albumin mg/l	UAC mg/g	USG	SDS-PAGE
40		194.2	0.03	49		0.48		1060	physiological
41	13	256.5	0.05	37	14.4	0.6	0.2	1050	physiological
42	102.7	303.5	0.34	48	15.8	1.05	0.4	1050	physiological
43			0.07	33		2.35		1045	physiological
44	7.1	177.7	0.04	32	18	6.11	3.4	1040	physiological
45			0.09	67		14.88		1060	physiological
46	23.4	200.1	0.12	45	22.5	16.54	8.3	1048	physiological
47			0.11	28		0.6		1028	physiological
48	5.5	80.2	0.07	27	33.8	2.06	2.6	1034	physiological
49	14	150.6	0.09	27	18	6.65	4.4	1060	physiological
50	31.7	320.4	0.1	31	9.7	2.73	0.9	1046	physiological
51	12.7	157.8	0.08	37	23.4	4.28	2.7	1028	physiological
52	25.3	375.3	0.07	36	9.6	0.83	0.2	1060	physiological
53	32.4	343.3	0.09	34	10	3.65	1.1	1050	physiological
54			0.16	38		0.42		1042	physiological

Table 24: Urinalysis results for the healthy control dogs and the diseased group on day 0: median, range and significance of difference

	Protein mg/dl	**Creatinine** mg/dl	**UPC** mg/mg	**RBP** µg/l	**URBPC** µg/g	**Albumin** mg/l	**UAC** mg/g
Healthy controls	18.7 5.5 - 103	228 80.2 - 375	0.09 0.03 - 0.34	36.5 27 - 67	18 9.6 - 33.8	2.35 0.42 - 16.54	1.9 0.2 - 8.3
Diseased dogs	71.8 3.6 - 1361	88.8 8.2 - 520	0.65 0.09 - 7.94	122 29 - 205	110 22.6 - 788	32.8 2.4 - 530	46.1 3.1 - 1388
Significance	p<0.001	p<0.01	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
Survivors	76.8 3.6 - 592	76.5 8.2 - 420	1.08 0.9 - 7.94	109 29 - 205	147 22.6 - 788	32.8 2.4 - 530	60 3.1 - 1338
Non-Survivors	60.3 18.8 - 1361	145 34.8 - 520	0.6 0.11 - 3.82	126 85 - 188	85.6 29.1 - 537	37.5 10 - 491	38.6 5.6 - 225.2
Significance	NS	p<0.05	NS	NS	NS	NS	NS

NS=not significant

9.2 List of tables

9.3 List of figures

List of publications

Schaefer H, Kohn B, Schweigert FJ, Raila J: Quantitative and qualitative urine protein excretion in dogs with severe inflammatory response syndrome. J Vet Intern Med. 2011 Nov; 25(6): 1292-7.

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Selbständigkeitserklärung:

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Helen Schaefer

Adelaide, Australien, den 18.12.2010