Department of Equine and Small Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

POTENTIAL URINARY BIOMARKERS OF ACUTE KIDNEY INJURY IN DOMESTIC ANIMALS

Mari Palviainen

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Veterinary Medicine of the University of Helsinki, for public examination in lecture room 1041, Viikinkaari 5 (Biokeskus 2), Helsinki, on March 28th, 2014, at 12 o'clock.

Supervisors

Professor Outi Vainio, DVM, PhD, Dipl ECVPT
Department of Equine and Small Animal Medicine,
Faculty of Veterinary Medicine,
University of Helsinki, Finland

Docent Marja Raekallio, DVM, PhD
Department of Equine and Small Animal Medicine,
Faculty of Veterinary Medicine,
University of Helsinki, Finland

Sami Junnikkala, PhD
Department of Veterinary Biosciences,
Faculty of Veterinary Medicine,
University of Helsinki, Finland

Reviewers

Professor Joseph Bartges, DVM, PhD, DACVIM, DACVN
Department of Small Animal Clinical Sciences
College of Veterinary Medicine
University of Tennessee
USA

Professor Antonia Vlahou, PhD
Biomedical Research Foundation
Academy of Athens
Greece

Opponent

Docent Aaro Miettinen, MD, PhD
Department of Bacteriology and Immunology,
Haartman Institute,
University of Helsinki, Finland

ISBN 978-952-10-9796-6 (paperpack)
ISBN 978-952-10-9797-3 (PDF)

Unigrafia Oy
Helsinki 2014

I may not have gone where I intended to go, but I think I have ended up where I needed to be.
Douglas Adams

CONTENT

1 ABSTRACT

Acute kidney injury (AKI) is a rapid loss of kidney function, which can be a consequence of ischemic, toxic or obstructive insult to the tubules, a reduction of the filtering capacity of the glomeruli or tubulointerstitial inflammation. The diagnosis and prognosis of AKI are problematic due to the lack of sufficiently specific and sensitive markers. The aims of these studies were to assess how kidney impairment impacts on the urinary proteome and to reveal the pathophysiological processes in kidney tissue caused by toxic insult. Changes in the urine proteome after toxic insult to the kidneys were studied by measuring urine enzyme activities, total protein and creatinine concentrations, and using proteomic methods. The usefulness of urinary enzyme activities in kidney impairment diagnosis was studied with in dogs bitten by *Vipera berus berus* (common European adder) and in sheep with ketoprofen-induced AKI. In both cases, the urinary enzyme activities were demonstrated to be promising markers of kidney impairment.

Two-dimensional gel electrophoresis (2D-GE) and two-dimensional differential gel electrophoresis (2D-DIGE) were used to detect potential new urinary protein markers in diagnosing AKI resulting from intoxication in sheep and dogs. The detected differentially expressed proteins were identified using a peptide mass fingerprinting method with either a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF) or a liquid chromatograph hybrid quadrupole mass spectrometer (LC-MS/MS). As a result, several potential urinary markers of kidney impairment were identified: retinol binding protein 4, calbindin D28k, CD1d, complement C3 and complement C4 in our sheep model of AKI, and alpha-1-antirypsin, β-2-microglobulin, fetuin-B and superoxide dismutase (Cu-Zn) in dogs bitten by the common European adder. The pathophysiological process in kidney tissue in renal impairment was examined using immunohistochemical methods. The possible involvement of calbindin D28k, CD1d and complement (C) components in the pathophysiology of AKI in our sheep model was also investigated. All antigens were localized in kidney tubule epithelial cells and the tubular lumina of the exposed sheep, confirming acute tubular injury detected by histological stains.

In summary, the measurement of urine enzyme activities proved to be an efficient method to evaluate kidney function in two domestic animal species. Several potential urinary markers were found, and warrant further investigation in clinical veterinary patients suffering from kidney impairment. The complement system was activated in kidney tissue in the ketoprofen-induced sheep model of AKI.

2 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

I Raekallio, M., Saario-Paunio, E., Rajamäki, M.M., Sankari, S., Palviainen, M., Siven, M., Peltoniemi, M., Leinonen, M-E., Honkavaara, J., Vainio, O. Early detection of ketoprofen-induced acute kidney injury in sheep as determined by evaluation of urinary enzyme activities. Am J Vet Res 2010: 71: 1246–1252.

II Palviainen, M., Raekallio, M, Rajamäki, M. M., Linden. J., Vainio, O. Kidney-derived proteins in urine as biomarkers of induced acute kidney injury in sheep. Short communication. Vet J 2012193: 287-289.

III Palviainen, M., Junnikkala, S., Raekallio, M., Meri, S., Vainio, O. Activation of complement system in kidney after ketoprofen-induced kidney injury in sheep. Submitted for publication 2014.

IV Palviainen, M., Raekallio, M., Vainionpää, M., Kosonen, S., Vainio, O. Proteomic profiling of dog urine after European Adder (*Vipera berus berus)* envenomation by two-dimensional difference gel electrophoresis. Toxicon 2012: 60: 1228–1234.

V Palviainen, M., Raekallio, M., Vainionpää, M., Lahtinen, H., Vainio, O. Evaluation of kidney impairment after *Vipera berus berus* envenomation in dogs. Short communication. Vet J 2013, doi 10.1016/j.tvjl.2013.09.008.

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3 ABBREVIATIONS

1D-GE	One-dimensional gel electrophoresis
2D-DIGE	Two-dimensional difference gel electrophoresis
2D-GE	Two-dimensional gel electrophoresis
β_2MG	β_2-Microglobulin
AAT	Alpha-1-antitrypsin
ACP	Acid phosphatase
AKI	Acute kidney injury
ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
ATI	Acute tubular injury
BSA	Bovine serum albumin
C	Complement
CNS	Central nervous system
COX	Cylooxygenase
DAB	3,3′-Diaminobenzidine
FABP	Fatty acid-binding protein
ELISA	Enzyme-linked immunosorbent assay
ESRF	End-stage renal failure
GFR	Glomerular filtration rate
GGT	Gamma-glutamyl transpeptidase
HMW	High-molecular-weight protein
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
IL-18	Interleukin-18
IMW	Intermediate-molecular-weight protein
IPG	Immobilized pH gradient
LC	Liquid chromatography
LDH	Lactate dehydrogenase
LMW	Low-molecular-weight protein
MALDI	Matrix-assisted laser desorption/ionization

MDRD	Modification of Diet in Renal Disease
MS	Mass spectrometry
NGAL	Neutrophil gelatinase-associated lipocalin
NSAID	Nonsteroidal anti-inflammatory drug
NKT	Natural killer T-cells
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid Schiff stain
PG	Prostaglandins
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
PGG2	Prostaglandin G2
PGH2	Prostaglandin H2
pI	Isoelectric point
PMF	Peptide mass fingerprinting
RBP4	Retinol binding protein 4
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD1	Superoxide dismutase 1
TBS	Tris-buffered saline
TOF	Time of flight

4 INTRODUCTION

Kidneys serve essential regulatory roles, notably urine secretion, in most vertebrates. In mammals, they are located in the abdominal cavity outside the peritoneum. Each kidney is surrounded by the renal fascia, a layer of connective tissue composed of a fibrous capsule and fat tissue known as perirenal fat. The parenchyma of the kidneys is formed of cone-shaped renal pyramids, the broad bases of which face towards the cortex. The urine-producing functional structures, the nephrons, are located through the cortex and medulla, and are composed of the glomerulus, proximal tubule, loop of Henle, distal tubule and collective duct. The glomerulus consists of a tuft of capillaries enveloped by spherical double-walled capsule (Bowman's capsule).

Urine, the secretion of kidneys, is a waste product of the body. However, urine is an ideal biological sample for the discovery of biomarkers, as it is easy to collect without any invasive procedures. Urinary proteomics is one of the fastest growing subdisciplines of proteomics in biomedical research (Thongboonkerd, 2010), and it is used not only in kidney disease biomarker research but also in investigating other organ disorders and systemic diseases in humans, such as diabetic nephropathy (Rao et al., 2007), bladder cancer (Goodison et al., 2009), colorectal liver metastasis (Bröker et al., 2013) and atherosclerosis in humans and mice (von zur Muhlen et al., 2012). As urine is a result of the filtration of plasma, its contents can vary depending on the primary reason for kidney impairment. It is important to understand which urinary proteins could serve as generic markers for kidney impairment, preferably distinguishing glomerular and tubular proteinuria from one another, and which markers are specific to a certain disease and reflect the general state of the individual.

In proteomic studies it is important that the results are interpreted with caution, as certain proteins seem to predominate the list of differentially expressed proteins in the majority of studies, irrespective of the disease or species (Petrak et al., 2008). Biomarker identification, validation and implementation are challenging (Mischak et al., 2010; Mischak et al., 2012). The bottleneck in the biomarker pipeline is the validation step with other diseases to determine the specificity and sensitivity of the marker to the target disease. The majority of

published proteomic studies have involved small single-centre trials, while the validation step requires larger trials with heterogeneous study populations. The majority of interesting novel urinary biomarkers of acute kidney injury (AKI) are low-abundance proteins discovered using effective proteomic methods. In clinical settings, the use of these laborious and expensive methods is problematic, and there is a demand for more cost-effective methods that yield rapid results.

Methods based on antibodies, such as enzyme immunoassays (EIA), are used to validate the results from the proteomic approach. The majority of proteins are subjected to alternative splicing and post-translational modifications, altering the composition of isoforms. Antibodies usually recognize particular epitopes of a protein, and if only a particular isoform of a protein is relevant to a certain disease, validation with immunological methods can fail. A combination of several markers appears to have greater diagnostic and predictive capabilities in humans than any single biomarker (Han et al., 2008; Vaidya et al., 2008; Metzger et al., 2010; Hall et al., 2011).

Intoxications caused by a snake bite or an accidental overdose of NSAIDs are common in domestic animals, especially dogs. These accidents can lead to AKI, a life-threatening condition in which early diagnosis is problematic and mortality is high. AKI is generally defined as a decline in renal function resulting in an accumulation of nitrogenous and non-nitrogenous waste products in the blood circulation (Venkataraman and Kellum, 2007). It is usually caused by an ischemic or toxic insult that results in damage to the proximal tubules (Silva, 2004). In ischemic-reperfusion AKI, both innate and adaptive immune systems are active and contribute to pathophysiological changes in the kidney tissue (Kinsey et al., 2008). Routinely used parameters, such as serum creatinine and urea concentrations, are insensitive and non-specific to acute changes in kidney function and kidney injury. They have a minor prognostic value in AKI, which is a genuine problem in clinical veterinary medicine. The early detection of AKI is essential for effective treatment and the prognosis, but the traditional laboratory approach for AKI, such as measuring serum creatinine and urea concentrations, generates a time gap between injury and a significant increase in these indicators. Thus, sensitive, early biomarkers are needed to detect those patients with or at risk of AKI to enable early intervention and the prevention of injury. Biomarkers may also serve several other purposes, such as identifying AKI aetiologies and

predicting AKI severity. The ideal biomarker would allow the discrimination of patients at risk of AKI before the insult to kidney tissue and true AKI patients. For clinical use, biomarkers should be straightforward to measure from samples that are easily accessible. Several published studies have revealed novel urinary biomarkers associated with various kidney diseases in humans and animals (Metzger et al., 2010; Varghese et al., 2010; Nabity et al., 2011; Sirota et al., 2013).

The research presented in this thesis suggested and tested potential urinary markers for assessing kidney impairment after certain intoxications in domestic animals before the routinely used parameters react.

5 REVIEW OF THE LITERATURE

5.1 THE EXCRETION AND ABSOBTION OF URINARY PROTEIN

The main function of glomeruli in the kidney is to filter low-molecular-weight substances from the blood to the urine and at the same time retain larger macromolecules in the blood flow. Filtered substances have to pass three barriers in the glomerulus, which together form a highly selective sieving filter, before entering the tubular compartment: the endothelium of the glomerular capillaries, the glomerular basement membrane and podocytes. The first barrier, the endothelial cell surface of glomerular capillaries containing glycocalyx, is a plasma membrane-bound part of the barrier composed of glycoproteins, glycosaminoglygans (GACs) and proteoglycans (Rennke et al., 1975). The second barrier, the basement membrane, is a three-layer structure comprised of a heteropolymeric network of type IV collagen, laminin, fibronectin, entactin and heparin sulphate proteoglycans (Laurie et al., 1984). The third barrier consists of the epithelial cells covering the outer surface of the glomerular capillaries and facing Bowman's capsule, called podocytes. Between individual podocytes are filtration slits filled with glycocalyx (Kerjaschki, 1994). The endothelial glycocalyx contributes to the permeability of the capillary wall (Singh et al., 2007). Although the exact mechanism of glomerular passage of plasma proteins is still unclear, proteins are filtered from primary urine based on their size, weight and shape (Haraldsson and Sörensson, 2004).

The reabsorption of proteins takes place in the proximal tubule, where the epithelial cells represent an effective apical endocytic apparatus (D'Amigo and Bazzi, 2003). Abundant serum proteins, such as albumin, immunoglobulin light chain, transferrin, vitamin D-binding protein and myoglobin, are reabsorbed in the proximal renal tubules, mainly by the endocytic receptors megalin and cubilin (Maunsbach, 1966; Cui et al., 1996; Batuman et al., 1998; Burne et al., 1999; Christensen and Gburek, 2004). The reabsorbed proteins are transported inside vesicles to the endosomal compartment of the epithelial cells, and fused with

endosomes, where degradation of these proteins takes place. The degradation products, amino acids, are returned to the circulation (Carone et al., 1979).

Even though the protein concentration of urine from healthy individuals is 1000 times less than that of plasma, with proteomic methods approximately 1500 separate proteins have been detected in urine (Pieper et al., 2004; Adachi et al, 2006). In a study by Pieper et al. (2004), the urine proteins were separated into two sample groups according to their size before identification by mass spectrometry. Plasma-derived proteins constitute only one-third of the circa 420 identified proteins. A study by Adachi et al. (2006) revealed that urine from healthy humans contains an enriched portion of extracellular and, surprisingly, also plasma membrane and lysosomal proteins.

5.2 ACUTE KIDNEY INJURY

AKI is generally defined as a rapid loss of nephron function resulting in an accumulation of nitrogenous and non-nitrogenous waste products (Venkataraman and Kellum, 2007). The causes of AKI in humans include complex surgery, nephrotoxic medication, sepsis and non-renal disease, and in developing countries also diarrhoea, infections, animal venoms, septic abortion, dyes and natural medicines (Fortenberry et al., 2013; Li et al., 2013). In dogs and cats, common complications leading to AKI include ischemia, infarction, toxins, infectious disease, bladder rupture, hypercalcaemia, hyperviscosity, multiple organ dysfunction, sepsis, pneumonia and acute pancreatitis (Ross, 2011; Thoen and Kerl, 2011). The prognosis depends greatly on the cause of AKI in both humans and animals. The mortality rate in dogs is approximately 53% to 60% (Behrend et al., 1996; Vaden et al., 1997; Segev et al., 2008), and in cats 50% (Worwang and Langston, 2008).

AKI can evolve from ischemic, toxic or obstructive insult to renal tubules, tubulointerstitial processes with inflammation, or a reduced filtering capacity of the glomerulus (Thadhani et al., 1996). A lowered GFR followed by altered protein traffic in the kidney can induce tubulointerstitial inflammation and therefore contribute to the progression of kidney injury (Abbate et al., 1998). Tubular cells can be further damaged by various components, such as growth

factors, albumin and complement components in the proteinuric flow. Tubulointerstitial damage can lead to a decrease in GFR via several mechanisms. When damage occurs, it will 1) increase fluid delivery to the macula densa and affect the tubuloglomerular feedback system (Bohle et al., 1987), 2) lead to atubular glomeruli and therefore reduce the total number of functional nephrons (Marcussen 1992), and 3) lead to the elimination of postglomerular capillaries, resulting in ischemic renal injury (Nangaku, 2004). As a result of these processes, proteinuria develops.

5.3 PROTEINURIA

Renal proteinuria can result from glomerular or tubular impairment. Roughly speaking, the leakage of HMW proteins (e.g. >69 KDa) to urine indicates glomerular proteinuria resulting from disturbed glomerular filtration, and the leakage of LMW proteins (e.g. < 60 KDa) to tubular proteinuria resulting from disturbed protein reabsorption (Bazzi et al., 1997). Selective proteinuria, where IMW proteins, mainly albumin, are passed through the glomerular barrier together with LMW proteins, is a result of moderate changes in glomerular permeability (Joachim et al., 1964). Disturbance of the glycocalyx in the glomerulus leads to altered glomerular permeability and proteinuria via reduced a charge, and to some extent, size and selectivity (Jeansson and Haraldsson, 2003; Fridén et al., 2011; Salmon et al., 2012). Several studies have demonstrated that the glomerular pore distribution changes in developing proteinuria so that more HMW and IMW proteins pass through the glomerular barrier (Scandling and Myers, 1992; Ruggenenti et al., 1999; Bakoush et al., 2002; Lemley et al., 2002).

Proteinuria further promotes tubulointerstitial damage, inducing apoptosis, and therefore plays an essential role in the progression of end-stage renal disease (Remuzzi and Bertani, 1998; Thomas et al., 1999; Ohse et al., 2006). As a result of tubular injury, the excretion of a progressively larger fraction of LMW proteins is manifested (D'Amico and Bazzi, 2003). Work conducted with an *in vitro* model of tubular epithelial cells has implied that proteinuria activates inflammatory and vasoactive molecules, such as chemokine monocyte

chemoattractant protein-1 (MCP-1) (Wang et al., 1997; Zoja et al., 1998, Yard et al., 2001; Donadelli et al., 2003). Overload proteinuria in rats induces the production of MCP-1 in the renal tubules *in vivo*, suggesting that monocytes are recruited to the site of injury (Eddy and Giachelli, 1995). Complement components have also been detected in the urine in patients suffering from proteinuria, and complement activation has been thought to be at least partly responsible for tubular injury (Camussi et al., 1982; Camussi et al., 1985; Biancone et al., 1994; Khan and Sinniah, 1995; Morita et al., 2000). Astor et al. (2011) reported in human patients with chronic kidney disease that a low GFR together with albuminuria was associated with mortality and end-stage renal disease. In human patients with primary glomerulonephritis, the tubular proteinuria is more often associated with ESRF than in patients with glomerular proteinuria (Bazzi et al., 1997). Controversially, human patients with clinical AKI and glomerular proteinuria developed chronic or end-stage renal failure more often than patients with tubular proteinuria (Suhail et al., 2011). It appears that independently of the initial insult on the kidneys, the progressive process of parenchymal damage paves the way for terminal renal failure both in glomerular and tubular proteinuria (Remuzzi et al., 1997).

5.4 LABORATORY ASSESSMENT OF KIDNEY FUNCTION

5.4.1 BLOOD

The plasma creatinine concentration is probably the most widely used analyte to evaluate kidney function. Creatinine is a product of creatine and phospocreatine metabolism. It is freely filtered by the glomerulus and does not bind to proteins. Factors such as age, gender, muscle mass and hydration status can alter the creatinine concentration. In dogs, the plasma concentration of creatinine appears to be higher in sight hound breeds such as Greyhounds (Hilppö 1986; Freeman et al., 2003). Other factors that have been reported to alter the plasma creatinine concentration in dogs include living outside (Rautenbach and Joubert, 1988), the circadian rhythm (Sothern et al., 1993), the site of blood sampling (Jensen et al., 1994) and physical effort (Snow et al., 1988; Rose and Bloomberg, 1989; Hinchcliff et al., 1993). In human medicine, the GFR is calculated by measuring

serum creatinine and usually using either the Cockcroft-Gault or MDRD formula to estimate creatinine clearance (Cockcroft and Gault, 1976; Levey et al., 1999). In veterinary medicine, kidney function can be evaluated by two means: either measuring the plasma urea and creatinine concentration, or measuring the GFR by clearance methods using indicators such as inulin or iohexol (Gleadhill and Michell, 1996; Brown et al., 1996; Haller et al., 1998).

In human medicine, the RIFLE system is used to assess the severity of AKI based on the alteration in the serum creatinine concentration from the baseline or by measuring urine output (Hoste et al., 2006). The acronym RIFLE is derived from the terms risk, injury, failure, loss and end stage in relation to kidney function. Although veterinary medicine lacks such an established staging system, there have been a few attempts to develop such criteria. Segev et al. (2008) introduced a scoring system for dogs managed with haemodialysis based on multiple variables, including plasma creatinine. Thoen and Kerl (2011) proposed a novel staging system, Veterinary Acute Kidney Injury (VAKI), for dogs based on serum creatinine concentration changes from baseline samples, as seen in the RIFLE system. Table 1 summarizes the two existing staging systems based on serum creatinine: RIFLE in humans and VAKI in dogs.

Table 1. The RIFLE system for humans and VAKI system for dogs used to evaluate the severity of AKI based on alterations in the serum creatinine concentration from the baseline.

RIFLE	**Staging**		**VAKI**
-	-	0	<1.5-fold increase in S-crea
1.5-fold increase in S-crea	Risk	1	1.5-1.99-fold increase in S-crea
2-fold increase in S-crea	Injury	2	2-2.9-fold increase in S-crea
3-fold increase in S-crea OR Absolute creatinine ≥354 µmol/L	Failure	3	≥3-fold increase in S-crea OR Absolute creatinine >354 µmol/L
Total loss of kidney function > 4 weeks	Loss	-	-
Renal replacement therapy needed	End Stage	-	-

5.4.2 URINARY PROTEINS

Measurement of the urine creatinine concentration is not valid in itself to evaluate kidney function. As creatinine is freely and mostly evenly excreted to primary urine, it serves as a marker for urine dilution. Other parameters measured from urine are therefore expressed as creatinine ratios. The urine protein:creatinine ratio, together with the urine albumin:creatinine ratio, is used to screen for kidney impairment resulting from several conditions in humans, including preeclampsia (Durnwald and Mercer, 2003) and diabetic nephropathy (Rodby et al., 1995).

Sensitive and specific biomarkers are needed in kidney impairment diagnosis. Preferably, a biomarker of acute kidney injury should be able to 1) detect the injury at an early stage, 2) distinguish glomerular and tubular injury, 3) distinguish renal and nonrenal injury, 4) assess the severity of the injury and 5) assess the effect of treatment (Nguyen and Devarajan, 2008). To be practical in clinical settings, a biomarker should be easy to detect and the sample should be noninvasively obtained. Decramer et al. (2008) summarized the advantages and disadvantages of urine as a sample matrix for proteomic studies. As an advantage: 1) urine is easily available and can be repeatedly collected in large quantities, 2) the proteins are relatively stable, since endogenous proteolytic activity has taken its course at the time of voiding, 3) the LMW proteins are usually soluble and therefore suitable for proteomic analysis without extensive treatment, 4) for kidney impairment diagnosis, urine is in close contact with the site of injury. As a disadvantage, the urinary proteome varies widely, resulting from variations in the diet and fluid intake, metabolic or catabolic processes, circadian rhythms and exercise, as well as circulatory levels of various hormones (Decramer et al., 2008).

For the discovery of novel urinary biomarkers of kidney impairment, proteomics has opened up new possibilities. The proteome is a set of proteins expressed in a cell, tissue or organism at a given time (Pennington et al., 1997). Proteomics offers a systematic multivariate analysis of proteins to identify, quantify and assess them functionally (Peng and Gygi, 2001). Two different approaches are used in the detection of proteins. In the gel-based approach, the proteins are separated according to their size with 1D-GE, or their size and pI

with 2D-GE, a technique that allows the visualization of several thousands of proteins using a wide pH range IPG strip, and even more using overlapping narrow pH range IPG strips (Wildgruber et al., 2000; Westbrook et al., 2001). With 2D-DIGE, the differences between proteins can be quantified (Unlü et al., 1997). Interesting proteins are then identified using mass spectrometry. The gel-free approach utilizes high-throughput mass spectrometry, and no separation of proteins takes place before analysis. In a review by Aebersold and Mann (2003), a variety of gel-free methods were discussed.

In the literature, there are several potential urinary markers whose applicability for diagnosing AKI in humans and animals has been assessed. Several urinary enzymes excreted from the proximal tubules have been indicated to be a reliable choice for diagnosing renal impairment: GGT, ALP and NAG in humans (Bazzi et al., 2002; Westhyusen, 2003; Han, 2008) and dogs (Rivers et al., 1996; Heiene et al., 2001; Lee at al., 2012). The analysis of enzymes is usually methodologically straightforward and provides some prediction of the onset of impairment (Price, 1982; Westhuysen et al., 2003). Some LMW urinary proteins, such as β_2MG, α_1-microglobulin, RBP and cystatin C, together with IMW and HMW proteins such as NGAL, IL-18, osteopontin and FABP are considered as biomarkers of kidney impairment in both humans (Hei et al., 2008; Askenazi et al., 2012; Arthur et al., 2013; Park et al., 2013; Zheng et al., 2013) and dogs (Raila et al., 2003; McDuffie et al., 2010; Smets et al., 2010; Vinge et al., 2010; Monti et al., 2012; Nabity et al., 2012). Kidney injury molecule 1 (KIM-1) is one of the investigated urinary markers for kidney injury in humans, rats and mice (Ichimura et al., 1998), but no evidence of its usefulness in clinical veterinary medicine has been reported. The evidence for the existence of KIM-1 protein in dogs and cats is scarce, merely occurring at the transcriptional level (UniProt, 2013).

Using the proteomic method, several promising urinary markers for different kidney impairment conditions have been found in both humans and animals. Some of the conventional urinary markers, such as α_1-microglobulin, β_2-microglobulin, cystatin C, osteopontin and RBP, appear to be represented in these studies (Ho et al., 2009; Good et al., 2010; Varghese et al., 2010; Nabity et al., 2011; Bellei et al., 2012). Proteomics has been able to suggest novel urinary markers for kidney impairment, such as fetuin-A in a rat model as well as in

human patients (Zhou et al., 2006), fumarylacetoacetate hydrolase (FAH) in a rat model of 4-aminophenol-induced kidney injury (Bandara et al., 2003), hepcidin-25 in human AKI patients (Ho et al., 2009), β defensin-1 in children suffering from contrast-induced nephropathy (CIN) (Bennett et al., 2008) and chitinase 3-like protein 1 (CHI3L1) in septic AKI in a mouse model, as well as in human septic AKI patients (Maddens et al., 2012).

5.5 RENAL TOXICOLOGY

Toxins can cause different degrees of kidney impairment, starting from minimal alterations in kidney function manifested as isosthenuria and proteinuria. Mild damage may be completely reversible. More severe consequences of nephrotoxicity include acute kidney injury with a significantly decreased GFR and are manifested as oligouria or anuria, proteinuria, aminoaciduria and glucosuria. Many potential nephrotoxic substances are therapeutic agents or originate from environmental exposure. Renal vulnerability to nephrotoxins varies between individuals depending on patient-specific, kidney-specific and drug-specific factors (Table 2). Nephrotoxicants can affect different sites in the kidney, hindering renal function via vasoconstriction (Sawaya et al., 1991), altered intraglomerular haemodynamics (Prendergast and George, 1993), tubular cell toxicity (Pallet et al., 2008), interstitial nephritis (Handa, 1986; Bennett et al., 1996), crystal deposition in the tubular lumen (deSequera et al., 1996), thrombotic microangiopathy (Pisoni et al., 2001) and osmotic nephrosis (Visweswaran et al., 1997). In the histopathological examination of kidneys suffering from toxicological insult, the major changes appear to be localized in the renal tubules, including the degeneration or necrosis of tubular cells, swelling of the epithelium, the detachment of tubular epithelial cells from the basement membrane, loss of the brush border, thinning of the epithelium, lumina dilation, casts in the tubular lumen, and rupture of the tubular basement membranes (Solez et al., 1979; Lameire and Vanholder, 2000).

Table 2. Factors that increase the risk of toxicant-mediated acute kidney injury in humans, modified from Perazella 2009.

Patient-specific factors	Old age Pre-existing renal dysfunction Volume depletion Metabolic disturbances Immune response - genes Pharmacogenetics favouring drug toxicology Diabetes mellitus
Kidney-specific factors	High rate of blood delivery Increased toxin concentration in renal medulla and interstitium Reactive oxygen species (ROS) High metabolic rate of the loop of Henle Proximal tubular uptake of toxins
Drug-specific factors	Prolonged dosing periods and toxin exposure Potent direct nephrotoxicity Combination of toxins Overdose

5.6 ADVERSE EFFECTS OF NSAIDS

The two isoforms of cyclooxygenase (COX), COX-1 and COX-2, belong to the prostaglandin G/H synthase family and serve as enzymes that convert arachnidonic acid to PG (Vane et al., 1998). Constitutive COX-1 is expressed in most cell types, excluding erythrocytes, whereas inducible COX-2 is primarily expressed in response to inflammation in many tissues. In addition, COX-2 has a constitutional role in the gastrointestinal tract, as well as reproductive and renal tissues (Dinchuk et al., 1995; Miller, 2006; Little et al., 2007). COX-1 is the most abundant isoform in the kidney, whose localization varies between species. Localization in the collecting ducts, renal vasculature and papillary interstitial cells is common to all mammals (Kömhoff et al., 1997; Khan et al., 1998; Câmpean et al., 2003). The expression of COX-2 in the kidney is species-specific. In dogs and rats, COX-2 expression is focused on the thick ascending limb of the loop of Henle and macula densa (Harris et al., 1994; Khan et al., 1998). In the adult human kidney, COX-2 is expressed in the glomerular podocytes, parietal epithelial and smooth muscle cells, and medullary capillaries lacking from the

macula densa (Kömhoff et al., 1997; Therland et al., 2004). After release from the cell membrane phospholipids in response to acyl hydrolases, most notably phospholipase A2, AA acts as a precursor molecule for eicosanoids. The COX pathway converts AA to cyclic endoperoxidase PGG2, which is further transformed to another cyclic endoperoxidase, PGH2. These endoperoxidases are further transformed to different prostanoids, such as PGD2, PGE2 and PGF2α. Prostanoids play an important role in inflammation by inducing fever and hyperalgesia. COX-2-induced PGs also mediate inflammatory swelling and vasodilation, causing the principal signs of inflammation: redness, heat, swelling, pain and loss of function (Funk, 2001).

NSAIDs are a group of drugs that provide analgesic, antipyretic and anti-inflammatory effects, and are administered in both humans and animals with acute and chronic pain conditions. Many NSAIDs are chiral molecules, most of which are manufactured in a racemic mixture. NSAIDs are mostly weak acids and highly protein-bound in plasma. They are usually metabolized in the liver, and inactive metabolites are excreted in urine. The anti-inflammatory, antipyretic and analgesic actions of NSAIDs are principally mediated by the inhibition of PG synthesis at the cyclooxygenase level (Capone et al., 2004). NSAIDs can be classified by COX selectiveness into traditional NSAIDs (nonselective) and coxibs, which mainly affect cyclooxygenase-2 (COX-2). The selectivity of a given NSAID affects the prevalence of adverse effects (Layton et al., 2008; Patterson et al., 2008). NSAIDs that possess greater inhibitory action on COX-2 than COX-1 have been regarded to be more beneficial, since the synthesis of inflammatory PGs is hindered but the cellular protective action of COX-1 products is spared (Brune and Hinz, 2004). Some adverse effects of selective NSAID use have been reported in humans, such as an increased cardiovascular risk (Mukherjee et al., 2001) and an increased incidence of congestive heart failure (Ray et al., 2002).

The most prominent side effect of NSAIDs is gastrointestinal (GI) irritation and ulceration in humans (Wallace, 1997), dogs and cats (Stanton and Bright, 1989; Hinton et al., 2002) due to the inhibition of constitutive COX-1. Clinical signs include anorexia, depression, diarrhoea, vomiting, haematochezia and melena. Possible secondary manifestations of GI ulceration and bleeding include anaemia and hypoproteinemia in humans, which can be transient (Wilcox and Clark, 1997; Hreinsson et al., 2013). Renal side effects of NSAIDs in mammalians

can be functional (PG-dependent) or anatomic (PG-independent) by direct toxicity (Bennett et al., 1996; Silverman and Khan, 1999; Khan et al., 2002; Kovacevic et al., 2003; Stern et al., 2010). Since homeostatic PGs act as vasodilators in the kidney, their inhibition by NSAIDs can decrease renal perfusion, leading to acute renal vasoconstriction, medullary ischemia and, ultimately, AKI (Oates et al., 1988). The direct toxicity of NSAIDs is suggested to act through mitochondrial membrane permeability transition, which subsequently triggers cell death in kidney tissue (Mingatto et al., 1996; Uyemura et al., 1997; Hickey et al., 2001). The renal toxicity of NSAIDs is more common in dogs and rodents than primates (Bennett et al., 1996; Khan et al., 1998; Sellers et al., 2004). The reason for this could be the differences between species in renal COX-2 expression.

5.7 ENVENOMATION BY *VIPERA BERUS BERUS*

The common adder (*Vipera berus berus*) is geographically widely distributed throughout Europe and Asia (Weinelt et al., 2002; Karlson-Stiber et al., 2006), and is the only venomous snake in Scandinavia. Every year, many domestic animals are bitten by common adders. Viper venom is produced by modified salivary glands and stored in venom sacks near the fangs. The venom of the common adder is a yellow liquid that contains a mixture of proteins with toxic and enzymatic properties aiming to kill or immobilize the prey and assist in digestion. *Vipera* species share characteristics at the familial and generic level in their venom composition, although the exact composition of *Vipera berus berus* venom is unknown. Variations in venom composition can be due to seasonal (Gubensek et al., 1974), individual (Master and Kornalik, 1964; Tan and Ponnudurai, 1990; Georgieva et al., 2008) and geographical differences (Jayanthi and Gowda, 1988). In a study on *Calloselasma rhodostoma* (the Malayan pit viper), it was noted that the variation in venom composition can also be genetically inherited (Daltry et al., 1996). In a study in rabbits, *Vipera aspis* venom showed a terminal half-life of 12 h in the circulation, and most of the venom was therefore eliminated within three days of envenomation, but only a small percentage of the venom was found to be excreted in urine (Auderbert et al.,

1993). *In vitro* and *in vivo* studies have demonstrated that snake venom of different species can be detected in kidney tissue after envenomation, and in some cases has a direct nephrotoxic effect (Ratcliffe et al., 1989; Burdmann et al., 1993; de Castro et al., 2004; Mandal and Bhattacharyya, 2007; de Roodt et al., 2012). A study in which purified Russell's viper venom-factor X activator was injected into rats demonstrated that renal failure could be a result of intravascular clotting in the kidney microcirculation rather that direct toxic insult (Suntravat et al., 2011).

Snake venom L-amino acid oxidases (SV-LAOOs) are components in common adder venom that are believed to contribute to the venomous effect by inducing apoptosis and haemorrhaging, and by affecting platelets (Tan and Ponnudurai, 1990; Li et al., 1994; Suhr and Kim, 1996; Samel et al., 2006). SV-LAAO catalyses the oxidative deamination of L-amino acids, simultaneously promoting the release of hydrogen peroxidase. In addition to SV-LAAO, most *Vipera* venoms have phosphodiesterase, hyaluronidase, 5'-nucleotidase, phospholipase A_2 (PLA_2) and protease activity (Tan and Ponnudurai, 1990; Yukel'son et al., 1995).

From common adder venom, metalloproteases and serine proteases degrading fibrinogen, faxtor X activators, and bradykinin-releasing serine proteases have been identified (Siigur et al., 2002). Snake venom metalloproteases (SVMPs) participate in the haemorrhagic process, degrading endothelial cell surface proteins and extracellular matrix components (Fox and Serrano, 2005; Escalante et al., 2006). PLA_2s are lipolytic enzymes that are divided into secreted ($sPLA_2$s), cytosolic ($cPLA_2$), Ca^{2+}-independent ($iPLA_2$), and lipoprotein-associated ($LpPLA_2$) phospholipases A_2 (Burke and Dennis, 2009). Snake venom PLA_2s belong to the $sPLA_2$s, and they are further divided into two groups according to the position of cysteine residues in the sequence (Heinrikson et al., 1977). Snake venom serine proteases (SVSPs) catalyse the cleavage of covalent peptide bonds and therefore participate in digestion, the regulation of blood coagulation, the immune system and inflammation (Neurath, 1984).

Hyaluronidase hydrolyses the hyaluronan, a part of the extracellular matrix, and contributes to local and systemic envenomation, enabling the spread of the other venom components (Tu and Hendon, 1983, Girish et al., 2002). Phosphodiesterases (PDEs) hydrolyse phosphodiester bonds from

polynucleotides, contributing to the generation of purine nucleosides, which can have a role in causing profound hypotension (Russel et al., 1963). 5'-Nucleotidase cleaves a variety of ribose and deoxyribose nucleotides, being most active against adenosine monophosphate (AMP). Its main function is suggested to be the release adenosine and other purines, resulting in the inhibition of platelet aggregation (Aird, 2002). Factor X activators activate coagulation factor X via cleavage, leading to the rapid formation of blood clots (Suntravat et al., 2011).

In humans, the majority of reported snake bites cause no or very mild effects (Reading, 1996), and approximately 10% of cases turn out to be so-called dry bites, meaning bites without envenomation (Petite, 2005; Karlson-Stiber et al., 2006). Envenomation by *Vipera berus berus* can induce multiform symptoms. The most notable local symptoms in dogs (Lervik et al., 2010) and humans (Reading, 1996; Grönlund et al., 2003; Karlson-Stieber et al., 2006) are swelling, bruising and pain in the area of the bite. In more severe cases, systemic symptoms such as shock, cardiovascular and gastrointestinal symptoms, CNS depression, AKI and respiratory distress occur in humans (Grönlund et al., 2003; Karlson-Stiber et al., 2006) and dogs (Lervik et al., 2010; Pelander et al., 2010).

6 AIMS OF THE STUDY

The studies forming this thesis focused on finding and reviewing urinary markers associated with kidney impairment in domestic animals after a toxic insult resulting from exposure to an NSAID and common adder venom. The goal was to provide evaluative tools for kidney function assessment based on laboratory analysis available in most veterinary clinics.

The specific aims of these studies were:

1. To determine the changes in urinary enzyme activities after NSAID overdose in sheep and envenomation by *Vipera berus berus* in dogs (I, V).

2. To identify potential urinary markers associated with kidney impairment after a toxic insult resulting from NSAID and common adder venom in domestic animals (II, III, IV).

3. To evaluate whether the complement system has a role in renal tissue destruction after an overdose of NSAID in sheep (III).

7 MATERIALS AND METHODS

Study protocols I–III were approved by the Ethics Committee of the Viikki Campus at the University of Helsinki. The protocols for studies IV and V were pre-evaluated and approved by the Ethics Board of the Faculty of Veterinary Medicine at the University of Helsinki.

The methods used have been described in more detail in the respective publications (I–V).

7.1 ANIMALS AND STUDY DESIGN

7.1.1 SHEEP

For studies I, II and III, twelve female Finnish Landrace sheep were purchased from a commercial breeder. All the sheep were >18 months old and none of them were pregnant. The body weight of the animals ranged from 48.5 to 59.0 kg (mean 52.5 kg). The sheep were provided *ad libitum* access to good-quality hay and water before and during the experiment. The sheep were randomly allocated to a treatment or control group (n = 6 sheep per group). Sheep in the treatment group were intravenously administered an overdose of ketoprofen (30 mg/kg) at time point 0, which was equivalent to 10 times the therapeutic dose (3 mg/kg) for cattle. The sheep in the control group did not receive any injection, since at the time of the study it was considered that a placebo injection would not affect kidney function and was therefore unnecessary.

7.1.2 DOGS

For studies IV and V, privately owned pet dogs were used. A total of 32 dogs accidentally bitten by *Vipera berus berus* and treated in the intensive care unit at the Veterinary Teaching Hospital of the University of Helsinki were recruited. The inclusion criteria were a strong suspicion of a viper bite (the owner saw the dog being bitten or saw a viper close to the dog), and clinical signs of a viper bite

defined as swelling and bruising in the bite area, pain and discomfort. A total of 23 control urine samples were obtained from clinically healthy pet dogs. Eleven affected dogs and eight healthy controls were included in study IV, and all the recruited dogs were included in study V.

7.2 SAMPLES

7.2.1 BLOOD

Blood samples were collected into tubes containing lithium heparin (I, II, III), citrate (V), and into plain tubes (IV, V). Samples were immediately centrifuged (1300 *g* for 10 min) and plasma or serum was harvested. An aliquot of each sample was taken for clinical chemistry analysis, in which the concentrations of albumin, ALP, creatinine, GGT, total protein and urea (I, V), in addition to ACP and AST (I), were measured with a Konelab 30i analyser (Thermo Scientific). The rest of each sample was frozen at -80 °C for further use.

7.2.2 URINE

Sheep urine samples were collected before treatment and at two, four, six and eight hours after ketoprofen administration via a urinary catheter. The catheter was inserted just before the injection of ketoprofen, and was removed after 8 hours. At the end of the study the final urine sample was collected via cystocentesis immediately after the sheep had been euthanized. Dog urine samples were collected as single void samples. An aliquot of each sample was taken for clinical chemistry analysis, in which the concentrations of creatinine and total protein and the activities of ALP and GGT (I, V), in addition to LDH (I), were measured with a Konelab 30i analyser. The results are expressed as ratios to the urinary creatinine concentration. The rest of the sample was centrifuged (1300 *g* for 10 min) to remove cellular debris and frozen at -80 °C until analysed.

7.2.3 TISSUE

Kidney tissue samples were obtained from sheep (I, II, III) at the end of the study when the animals had been anesthetized with xylazine 0.2 mg/kg and ketamine 5 mg/kg IV and immediately thereafter sacrificed with T61. A part of the tissue samples was placed in neutral-buffered 10% formalin, further processed by embedding in paraffin and sectioned into 4 or 7 μm thick slices. A further part of the tissue samples was snap-frozen in liquid nitrogen and stored at -80 °C for cryostat sections.

7.3 ANALYTICAL METHODS

7.3.1 ANALYSIS OF KIDNEY TISSUE

7.3.1.1 Histological examination and immunohistochemistry

The tissue samples in study I were sectioned and stained with haematoxylin–eosin, van Gieson, PAS, and von Kossa stains for histological examination via light microscopy. Paraffin slides (II) or cryoslides (III) were used to detect antigens in kidney tissue. To visualize bound antibodies (Table 3), an indirect immunohistochemical procedure was used, combining a polymer technique or streptavidin-peroxidase complex technique and nickel-enhanced DAB chromogen. Negative control sections were processed in parallel without the primary antibody.

7.3.2 ANALYSIS OF URINE

7.3.2.1 SDS-PAGE and immunodetection

In studies I, II, III and IV, native PAGE or SDS-PAGE was used with polyacrylamide gels as described previously (Laemmli 1970). An equal amount of protein from each sample was loaded onto gels and proteins were separated by electrophoresis at 100 V for 2 h. Proteins were transferred to a PVDF membrane

using semidry blotting apparatus. Immunodetection was performed by incubating blots with the appropriate primary antibody (Table 3) in a buffer of 1X Tris buffered saline (TBS) plus 0.1% Tween 20 after blocking the membrane with 5% BSA for 1 h. Blots then were washed 3 times with 1X TBS plus 0.1% Tween 20 and incubated with a horseradish peroxidase-conjugated secondary antibody in 1X TBS plus 0.1% Tween 20 at the appropriate dilution for 3 h at room temperature. The washing procedure was repeated and after the third wash, proteins were visualized using an enhanced chemiluminescent substrate and the signal was detected with a Fuji LAS-3000 high-resolution CCD camera. In addition to the immunodetection of C3c (III), the detection of urinary C3d was performed from three AKI and four control sheep, C4c from two AKI and three control sheep, C5 from two AKI and three control sheep, C9 from two AKI and two control sheep, and C1q from three AKI and one control sheep. The results of these Western blots are presented in this thesis.

Table 3. Antibodies used in immunostaining and Western blotting

Primary antibody	Host	Application	Dilution	Source	Publication
MMP2	Mouse	WB	1:1000	Chemicon	I
MMP9	Goat	WB	1:1000	Santa Cruz	I
C1q	Rabbit	IHC, WB*	1:1000	Dako	III
C3c	Rabbit	IHC, WB	1:1500	Behring	III
C3d	Rabbit	IHC, WB*	1:1500	Dako	III
C4c	Rabbit	IHC, WB*	1:1500	Dako	III
C5	Goat	IHC, WB*	1:1000	Quidel	III
C9	Goat	IHC, WB*	1:1000	Quidel	III
Factor H	Rabbit	IHC	1:500	Quidel	III
Calbindin D28k	Mouse	IHC	1:1000	Abcam	II
CD1d	Mouse	IHC	1:1000	Abcam	II
SOD1	Rabbit	WB	1:1000	Abcam	IV
AAT	Mouse	WB	1:1000	Santa Cruz	IV
Fetuin-B	Mouse	WB	1:1000	Abcam	IV

Abbreviations: IHC Immunohistochemistry; WB Western blotting; * not published previously.

7.3.2.2 Proteomic methods

7.3.2.2.1 Two-dimensional gel electrophoresis

In study II, the pooled urine samples were purified and concentrated with HPLC (Applied Biosystems 400 HPLC, Applied Biosystems) with an RPC column (GE Healthcare) and programmable absorbance detector (Applied Biosystem 738A, Applied Biosystems). Subsequently, the concentrations of proteins in the samples were measured by using a 2D Quant Kit (GE Healthcare).

Proteins were analysed by two-dimensional gel electrophoresis (2DE) as described by O'Farrel (1975) as follows. An IPG strip (pH 3–10: 7 cm) (GE Healthcare) was used for isoelectric focusing. IPG strips were loaded with 7 μg of total protein. The samples were focused at a total of 12 000 volt-hours for 3 h. After focusing, the isoelectric strips were prepared for the second dimension gels by incubation in equilibrium buffer solution I (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% sodium dodecyl sulphate (SDS), 0.2% bromophenol blue, with added 10mg/ml dithiothreitol (DTT)) for 15 min. This was followed by equilibration in buffer solution II (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.2% bromophenol blue, supplemented with 25 mg/ml iodoacetamide) for another 15 min. The prepared IPG strips were then placed on 12% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) and sealed with overlay agarose (Bio-Rad). Electrophoresis was initiated at 50 V for 15 min, which was followed by 150 V for 90 min. Gels were silver-stained without aldehyde.

The images of protein spots were scanned (Scanner GS800, Bio-Rad Laboratories) and spot intensives were obtained using the image analysis software PDQuest (Bio-Rad Laboratories). The images were normalized according to the total density of the gel and by comparing spot intensities between the samples.

7.3.2.2.2 Two-dimensional difference gel electrophoresis

In study IV, urine samples were concentrated and desalted by centrifugal ultrafiltration using Amicon Ultra-0.5 10 K centrifugal units (Millipore). In previously unpublished study with the urine samples collected before treatment

and at two, four, six and 24 hours after treatment from four control sheep and four ketoprofen treated sheep, low-abundance urinary proteins in sheep urine were first enriched using a bead-based library of combinatorial peptide ligands (ProteoMiner, Bio-Rad) and then purified using a 2D Clean Up kit (GE Healthcare) before labelling. The samples were then precipitated with trichloroacetic acid-acetone and solubilized in 50 µl of labelling buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris). The samples were then labelled with Cy2, Cy3 and Cy5 dyes (CyDye DIGE Fluor minimal dyes, GE Healthcare) after adjusting the pH of each sample to 8.5 if needed. The dyes were added to the protein samples at a ratio of 50 µg of protein per 400 pmol dye. Cy3 and Cy5 dyes were used to label the individual samples that were being compared, and Cy2 was used for the internal standard consisting of equal amounts of each sample. The labelling reaction was incubated for 30 min on ice in the dark and quenched by adding 1 mM lysine to the reaction following 10 min of incubation as earlier. The labelled samples were pooled and separated by two-dimensional gel electrophoresis.

An IPG strip (24 cm, pH 3–10, nonlinear, GE Healthcare) was used for isoelectric focusing. Strips were rehydrated in 500 µl DeStreak rehydration solution containing 1% IPG buffer 3-10 NL (GE Healthcare) overnight at room temperature. Samples containing 150 µg of protein in total in 50 mM DTT, 4 mM tributylphosphine and 1% IPG buffer 3-10 NL were applied to the IPG strips with a cup-loading method near the acidic end of the strips. Isoelectric focusing was performed using IPGPhor (GE Healthcare) at 20 °C as follows: 3 h at 150 V, 3 h at 300 V, then linear ramping to 10 000 V and 10 000 V for 50 000 Vh, with the maximum current per strip being 75 µA. The isoelectric strips were then prepared for the second dimension gels by incubation for 15 min in equilibrium buffer solution I. This was followed by equilibration for another 15 min in equilibrium buffer solution II. The strips were then placed on 12% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) and sealed with overlay agarose (Bio-Rad). Electrophoresis was initiated at 50 V for 30 min, which was followed by 400 V for 3 h. The gels were scanned between low-fluorescence glass plates using an FLA-5100 laser scanner (Fujifilm). After scanning, the gels were silver stained. The gel images were analysed and statistically assessed using DeCyder 7.0 software (GE Healthcare).

7.3.2.2.3 Protein identification by mass spectrometry

Protein spots of interest were manually excised from the gels and digested in-gel using trypsin, allowing the peptides to be recovered. The peptides were then analysed by PMF. In study II, peptides were analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Autoflex MALDI-TOF MS, Bruker Daltonics). In study IV and unpublished study of low-abundant urinary proteins, MS/MS of peptides was performed on a hybrid quadrupole/TOF mass spectrometer with a Nanospray II source (QSTAR Elite, applied Biosystems, Foster City, CA). Proteins were identified using the local Mascot (Matrix Science, London, UK) against the in-house database of published mammalian sequences. The results were evaluated by considering the probability score, sequence coverage and correspondence between the estimated and the experimental pI and molecular weight.

7.3.3 ASSESSMENT OF *VIPERA BERUS BERUS* ENVENOMATION IN DOGS

7.3.3.1 Clinical gradation and kidney function score

In study V, the clinical gradation of severity of envenomation was conducted with grading scale of Audebert et al. (1992) with modifications by Petit (2005) for common viper envenomation in human patients (Table 4).

Table 4. Clinical gradation of common European adder envenomation in dogs by Audebert et al. (1992) with modifications by Petit (2005).

Grade	Envenomation	Clinical feature
0	Dry bite	Fang marks, no local signs
1	Minor	Local swelling, pain, no systemic signs
2	Moderate	Extensive swelling and/or moderate systemic signs
3	Severe	Immense swelling and severe systemic signs

The severity of the impact on kidney function of *Vipera berus berus* envenomation was assessed with a three-step grading scale based on laboratory findings (Table 5).

Table 5. Kidney function score based on laboratory findings of dogs bitten by the common European adder.

Grade	Laboratory findings
1	Serum albumin, urea and creatinine within reference range in all samples taken
2	Increased urine protein:creatinine ratio (>0.005) with slightly increased serum urea (8.8–16 mmol/L) and/or creatinine concentration (11–200 μmol/L) and/or decreased serum albumin concentration (<28 g/L)
3	Serum creatinine concentration >200 μmol/L at some time point

7.3.4 STATISTICAL ANALYSIS

Statistical analyses (I, V) were performed using PASW Statistic (IBM). The normality of each parameter was tested with the Shapiro-Wilk test, and logarithmic transformation was performed when necessary before statistical analysis to obtain normally distributed data. In study I, differences in parameters between groups at various time points were analysed using the Mann-Whitney *U*-test, and the Wilcoxon rank sum test was used in comparison. In study V, the Mann-Whitney *U*-test was used for comparison of nonparametric data, Pearson correlation was used for normally distributed data and Spearman rank correlation for nonparametric data. Significance was set at $P < 0.05$. In the 2D-GE study (II), statistical analysis was performed with PDQuest (Bio-Rad Laboratories) software using the Student's t-test. In the 2D-DIGE studies (IV, unpublished study), statistical analysis was performed with DeCyder (GE Healthcare) software, in which a paired analysis of variation (ANOVA) method was used that assigned statistical significance to the differences in normalized protein abundance between each group. Protein spots demonstrating a minimum 1.5-fold difference in average spot volume ratios between groups or time points and having a Student's t-test *P*-value of less than 0.05 were picked and identified.

8 RESULTS

8.1 NSAID OVERDOSE-INDUCED AKI

8.1.1 VERIFICATION OF AKI

The plasma concentrations of creatinine (I) and urea started increasing 4 h after injection and kept increasing until the end of the study. Plasma concentrations of creatinine exceeded the reference range of 6 to 8 h (median, 6 h) after ketoprofen treatment. The urinary total protein:creatinine ratio (I) started to increase 2 h after injection, indicating proteinuria. These changes were not detected in the control group. Histological examination of kidney tissue after haematoxylin–eosin, van Gieson, PAS, and van Kossa staining revealed ATI in NSAID-treated sheep (I). In particular, proximal tubules showed lesions, including degeneration and necrosis of individual tubular epithelial cells. Detachment of the tubular epithelium from the tubular basement membrane, loss of the PAS-positive brush border, thinning of the tubular epithelial cells and interstitial oedema together with cellular debris and casts in the distal tubules were also detected. None of the above was detected in control sheep kidney tissues. Accordingly, these findings were considered as a sign of AKI induced by an overdose of ketoprofen.

8.1.2 FINDINGS IN URINE

Table 6 summarizes the findings in urine measured with a clinical chemistry analyser (I). In addition to the results presented in Table 7, pro-MMP2 and active MMP2 increased in sheep with ketoprofen-induced AKI 6 h after administration. 2D-GE analysis (II) revealed five upregulated proteins in sheep urine after AKI compared to control sheep urine (Table 7). From these identified proteins, apolipoprotein A1 (ApoA-1) appeared within both groups by the 2 h time point and remained visible for the rest of the time points for both groups. Calbindin D28k and RPB4 appeared in the ketoprofen treatment group after 2 h and were

detectable at all later time points, being absent in the control group. CD1d appeared in the ketoprofen treatment group after 4 h and was detected at all subsequent time points, being absent in the control group. In addition, haemoglobin was detected and identified in the urine of AKI sheep 2 h after the administration of ketoprofen. In unpublished 2D-DIGE analysis, three upregulated proteins were identified (Table 7, figure 1). The MASCOT search identified C3 and C4 as their complete forms, but the measured MWs were lower than the theoretical ones. This difference can be explained by the enzymatic cleavage of their inactive forms C3β and C4γ. Peptide fragments and amino acid sequences established by MS/MS analyses that indicate the enzymatic cleavage products are shown (figure 2). In Western blot analysis of urine (III), excretion of C3c was detected in both groups. The excretion patterns were similar in case and control sheep, showing intact C3 (~190 kDa) and its degradation products C3b and iC3b (~180 kDa), C3c (~140 kDa), C3α´75 kDa (~75 kDa) and C3dg (~50 kDa). The excretion patterns of C1q, C3d, and C4c were similar in case and control sheep in Western blot analysis (Figure 2). The detected protein band in the anti-C1q immunoblot was relatively large (~200 kDa), and was above the theoretical size of the C1q protein (~40 kDa), suggesting that the detected C1q was bound to other proteins. The detected protein bands in the anti-C3d immunoblot matched the intact C3 (~190 kDa), and the degradation products C3b (~170 kDa) and C3d (~40 kDa). Anti-C4c detected the intact C4 (~192 kDa), C4b (~80 kDa) and C4c (~75 kDa) (Figure 3).

Table 6. Median (range) values for the urinary concentration of creatinine, the protein:creatinine ratio, and enzyme activity indices in samples collected from 6 sheep with ketoprofen induced AKI and from 6 control sheep.

Variable	Group	0 h	2 h	4 h	6 h	8 h	24 h
Creatinine (mmol/L)	Ketoprofen	17.8 (8.0–21.8)	10.9 (3.7–16.6)	7.2 (4.5-10)*†	3.9 (2.2–7.9)*†	2.7 (1.4–6.5)*†	2.7 (2.2–3.5)†
	Control	12.4 (8.9–14.9)	13.4 (10.9–25.4)	14.9 (6.3–27.1)	15.3 (12.7–25.6)	12.8 (6.6–24.8)	9.1 (2.1–20.0)
Protein:creatinine ratio (g/g)	Ketoprofen	0.11 (0.09-0.18)	0.96 (0.56-1.50)†	7.72 (4.09-19.6)*†	18.0 (6.69-63.1)*†	27.2 (3.05-112.9)*†	3.76 (0.97–16.5)†
	Control	0.14 (0.08–0.38)	0.49 (0.18–1.38)†	0.78 (0.34–1.77)†	1.41 (0.38–2.67)†	1.36 (0.69–3.58)†	0.77 (0.23–3.60)†
GGT (U/mmol)	Ketoprofen	0.86 (0.50–1.18)	0.86 (0.79–1.38)	1.66 (0.49–4.20)	3.02 (1.27–8.17)*†	6.17 (2.62-17.53)*†	2.62 (0.89–16.13)
	Control	0.94 (0.56–1.14)	0.81 (0.39–1.98)	1.01 (0.37–2.95)	1.07 (0.56–3.69)	1.34 (0.40–3.35)	1.33 (0.78–2.93)
ALP (U/mmol)	Ketoprofen	3.2 (1.9–5.0)	4.3 (1.9–9.7)	13.9 (9.4–42.4)*†	42.3 (27.7-135.8)*†	98.2 (16.2–420.8)*†	27.4 (8.7–86.3)†
	Control	3.7 (2.6–7.4)	4.3 (1.6–10.6)	5.2 (1.4–13.2)	5.8 (2.5–18.6)	9.6 (3.0–20.1)	8.5 (4.4–9.9)
ACP (U/mmol)	Ketoprofen	8.3 (7.3–9.1)*	13.9 (9.9–22.2)*†	26.8 (11.9–39.2)*†	34.4 (11.6–53.8)*†	30.9 (9.3–56.5)*†	9.5 (8.7–20.5)*†
	Control	7.1 (6.8–8.1)	7.2 (5.7–7.7)	5.9 (4.6–6.4)†	6.3 (5.3–6.8)	6.1 (5.8–8.1)	7.5 (7.1–9.6)
LDH (U/mmol)	Ketoprofen	0.95 (0.12–2.77)	5.98 (2.67–22.64)*†	51.05 (0–150.46)†	87.7 (40.2–228.2)*†	185.2 (28.2–535.4)*†	105.0 (39.9-305.7)*†
	Control	0.89 (0–4.36)	1.67 (0–2.19)	2.05 (0.97–2.69)	2.44 (0.68–6.88)	1.71 (1.09–17.60)	8.09 (0–59.33)
NAG (U/mmol)	Ketoprofen	0.03 (0.02–0.04)	0.12 (0.04–0.26)*†	NM	0.73 (0.17–1.40)*†	NM	0.26 (0.05–0.60)*†
	Control	0.03 (0.02–0.04)	0.02 (0.02–0.03)	NM	0.02 (0.02–0.04)	NM	0.04 (0.02–0.15)

*Within a time point within a variable, value differs significantly ($P < 0.05$) from the value for the control sheep. †Within a row, value differs significantly ($P < 0.05$) from the value for baseline.

Table 7. Statistically significantly ($P < 0.05$) upregulated proteins in the urine of ketoprofen-overdosed sheep compared to controls detected by 2D-GE coupled with MALDI-TOF-MS (II) and 2D-DIGE coupled with LC-MS/MS (unpublished data).

	Identified protein	Accession number	Theoretical pI/MW (Da)	Matched peptides	Sequence coverage (%)	MASCOT score	Ratio
2D-GE	Apolipoprotein A1	P15497	5.71/28.4	11	35.2	106	NM
	Calbindin D28k	P04467	4.7/29.9	4	19.2	51	NM
	Retinol-binding protein 4	P18902	5.44/21.4	6	37.7	78	NM
	CD1d	gi218931261	6.09/38.4	4	30.7	54	NM
	Haemoglobin beta subunit	gi164448674	6.49/1603	9	46.2	115	NM
2D-DIGE†	Apolipoprotein A1	P15497	5.71/28.4	38	64.15	636	4.39*
	C3β	Q2UVX4	8.96/71.4	2	2.95	51	4.63*
	C4 γ	P01030	6.68/32.9	2	7.21	96	4.72*

*Calculated between 2 h/ 0 h
†Unpublished

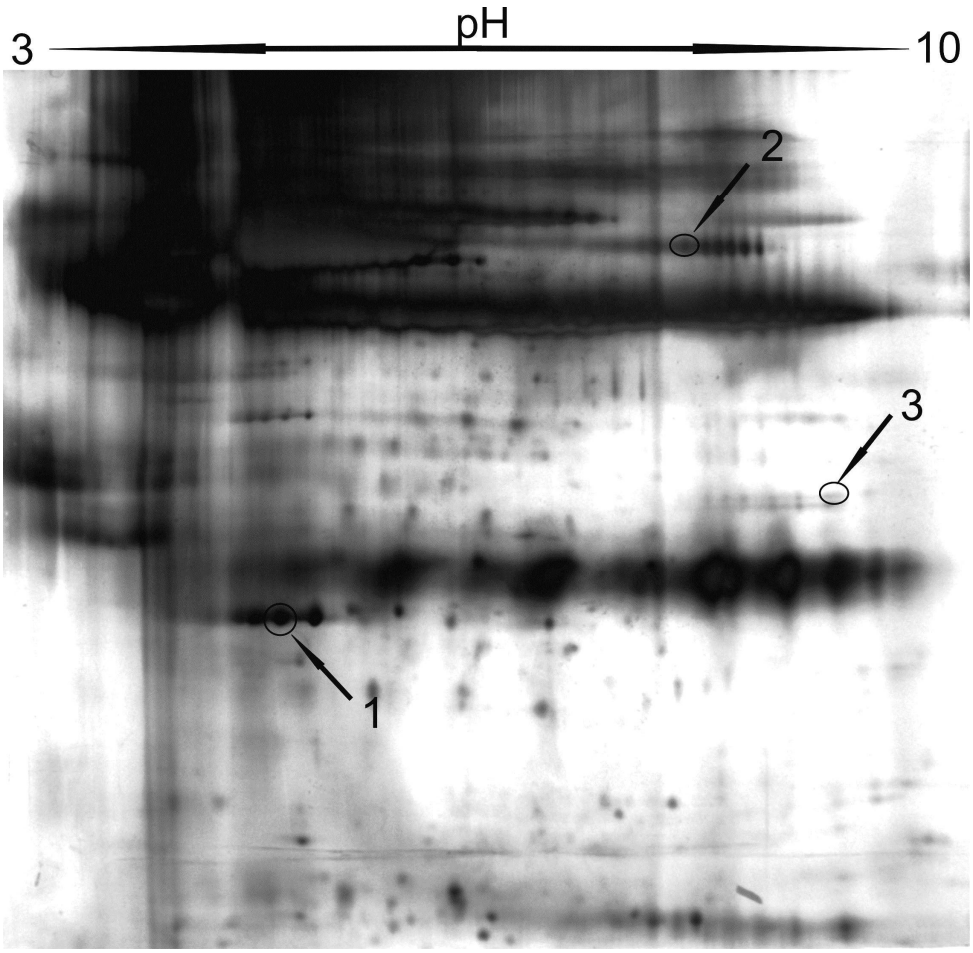

Figure 1. A representative image of a 2D-DIGE gel after silver staining showing protein spots successfully identified with LC-MS/MS that were significantly differently expressed between time points. Protein spots are marked by arrows and numbered: 1. Apolipoprotein A-1; 2. Complement component C3 β; 3. Complement component C4γ.

A

```
1    mkptsgpsll llllaslpma lgn
23      pmysmit pnilrlesee tvvleahggq gtiqvsvtvh
61   dfpakkqvls nentqlnsnn gylstvtiki paskelksdk ghkfvtvvat fgnvqvekvv
121  lislqsgylf iqtdktiytp gstvlyrvft vdhkllpvgq tvfitietpd gipvkrdsks
181  sqnqfgiltl swnipelvnm gvwkikayye dspqqvfsae fevkeyvlps fevqlepeek
241  fyyiddpdgl kvniiarfly geqvdgtafv ifgvqdgdrr islthsltrv pindgngeai
301  lkrqvllngv qpsradalvg ksiyvsatvi lqsgsdmvea ertgipivts pyqihftktp
361  kffkpampfd lmvyvtnpdg sparhipvvt qgsnvqsltq ddgvaklsin tqnkrdplti
421  tvrtkkdnip egrqatrtmq alpyntqgns nnylhlsvpr velkpgetln vnfhlrtdpg
481  eqakiryyty mimnkgkllk vgrqyrepgq dlvvlpltit sdfipsfrlv ayytlinakg
541  qrevvadsvw vdvkdscmgt lvvknggkee khhrpgqqit lkieadqgar vglvavdkgv
601  fvlnkknklt qrkiwdvvek adigctpgsg rnyagvftda gltlktsqgl etqqradpqc
661  pqpat
666       rrrrs vqlmekrmdk agqyssdlrk ccedgmrdnp mkfpcqrraq filqgdacvk
721  afldcceyit qlrqqhsrdg alelarsdld ddiipeedii srsqfpeswl wtviedlkqa
781  dkngistklm nvflkdsitt weilavslsd kkgicvadpy evtvmqdffi dlrlpysvvr
841  neqveirail ynyreaenlk vrvellynpa fcslatakkr hqqtitipar ssvavpyviv
901  plkiglheve vkaavynhfi sdgvkktlkv vpegvrvnkt vavrtlnpeh lgqggvqree
961  vpaadlsdqv pdtesetkil lqgtpvaqmt edaidgerlk hliqtpsgcg eqnmigmtpt
1021 viavhyldst dqwekfglek rqeslelirk gytqqlafrq kssayaafqy rppstwltay
1081 vvkvfalaan liaidskdlc etvkwlilek qkpdgifqed gpvihqemig gfrdtrekdv
1141 sltafvlial heakdiceaq vnslgrsiak agdflenhyr elrrpytvai aayalallgk
1201 legdrltkfl ntakeknrwe epnqklynve atsyallall arkdydttpp vvrwlneqry
1261 ygggygstqa tfmvfqalaq yqkdvpdhke lnldvsiqlp srnsavrhri lwesasllrs
1321 eetkenerft vkaegkgqgt lsvvtvyhak lkgkvsckkf dlrvsirpap etvkkpqdak
1381 gsmildictk ylgdqdatms ildismmtgf spdvedlktl stgvdryisk yemnrdsnkn
1441 tliiyldkvs htvedclsfk vhqyfnvgli qpgavkvysy ynldetcirf yhpdkedgml
1501 sklchkdtcr caeencfmhh tekevtledr ldkacepgvd yvyktrliqk kleddfdeyi
1561 mvieniiksg sdevqvkqer kfishikcre alklkegahy lvwgvssdlw gekpkisyii
1621 gkdtwvelwp eaeecqdeen qkqcedlanf tenmvvfgcp n
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B

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1   nvnfqkaihe klgqytspva krccqdgltr lpmartceqr aarvqqpacr epflsccqfa
61  eslrkkartr gqvglarvgf svvpiaaaav slkvvargsf dfpvgdaisk ilqveregal
121 hreemvyeln pldplgrtle ipgnsdpnii pegdfksfvr vtasdpleal gsegalspgg
181 lasllrlpqg caeqtmtlla ptlaasryld kteqwsmlpp etkdravdli qkgytriqef
241 rkrdgsygaw lhrdsstwlt afvlkilsla qdqvxgsaek lqetatwlls qqrddgpfhd
301 pcpvihremq gglvgsdetv altafvvial hhglavlpdk nsrvensisr antflgakat
361 sgllgshasa itayalslte apedlrrvah nnlmamakdi gdklywgsvt tspsnvlspt
421 paprspadpi pqapamsiet taygllhlll wegkaeladq aaswltrqgs fqggfrstqd
481 tvvaldalsa ywiasytaee kglnvtlssl grsglkshvl qltnhqvhrl eeelqfslgs
541 kinvevrgns kgtlkvlrsy nvmdmtnttc qdlqievtvm ghveytmeae edyedyeyed
601 llagddpeah srxvtplqlf dgrrnrrrr
630          e apkaaeeres rvqytvciwr tgkvglsgma
661 iaditllsgf halradlekl tslsdryvsh fetegphvll yfdsvptsre cvgfgavqev
721 pvglvqpasa ilydyynpeh kcsvfygapr kskllstlcs advcqcaegk cprqrraler
781 gqqdlegyrm kfacysprvd ygfqvkvlre dsraafrlfe tritqvlhft kdagatadqt
841 rnflvrascr lqlepgkeyl imgldgatyd lkgdpqylld snswieemps ermcqstrhr
901 tpcaqlnsfl qeygtqxcq
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Figure 2. Peptide fragments identified by MS/MS and sequence coverage of complement C3 and C4. Complement C3 (A) and complement C4 (B) amino sequences in one-letter code. C3 β chain (23-665) and C4 γ (630-920) are framed with a box. Peptides identified by peptide mass fingerprinting are highlighted in bold and underlined.

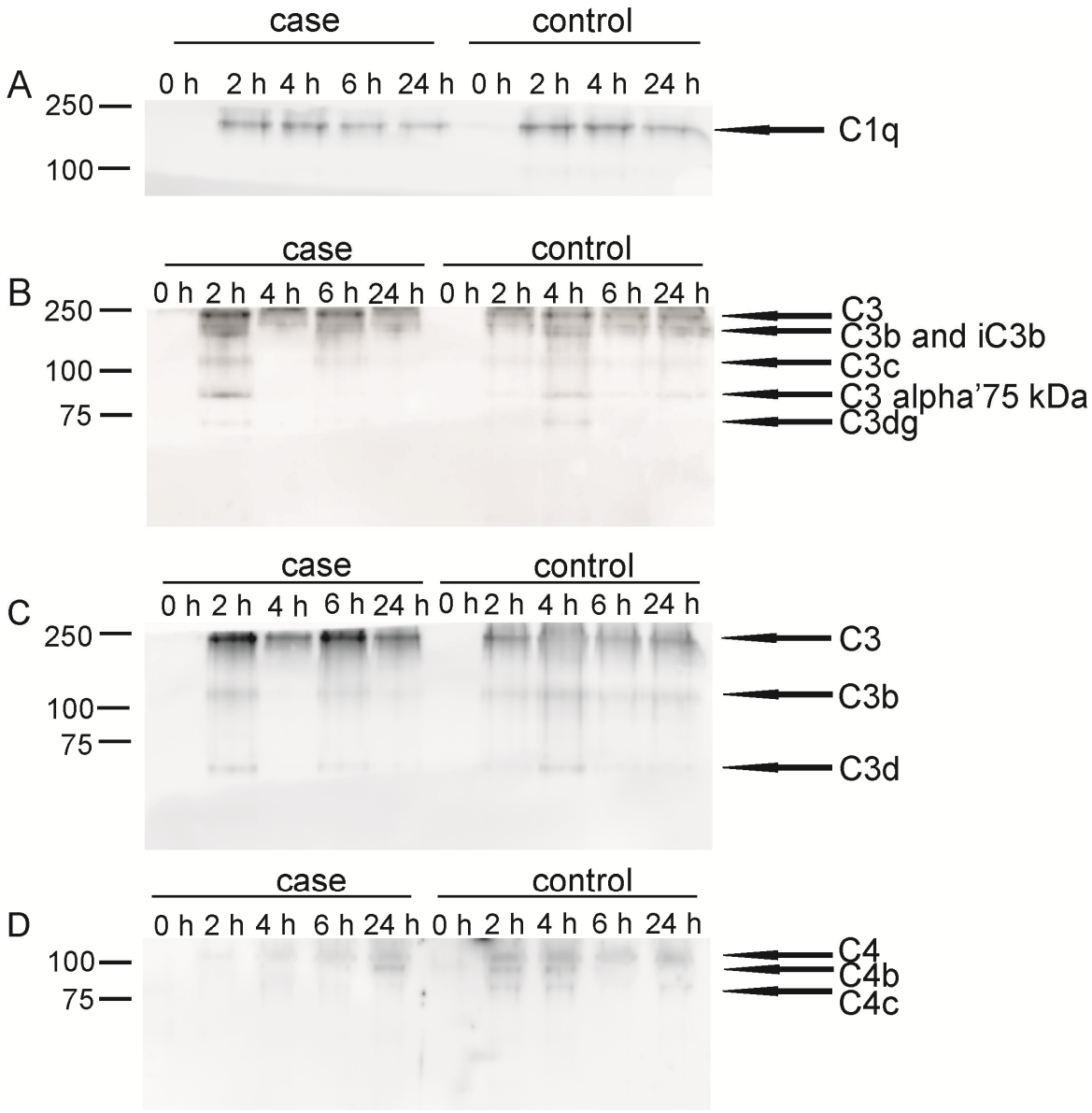

Figure 3. A representative image from Western blot analysis of the urine of control and ketoprofen-induced AKI sheep. The proteins were separated on 12% SDS-PAGE in non-reducing conditions and detected using polyclonal anti-C1q (A), anti-C3c (B), anti-C3d (C) and anti-C4c (D).

8.1.3 FINDINGS IN BLOOD

Table 8 summarizes the findings in blood (I). Plasma ACP activity was increased after administration of ketoprofen at the 8 h time point compared to control sheep. In addition to these variables, the serum calcium concentration was significantly lower 24 h after ketoprofen administration than in control sheep (II). A decrease was noted in the concentrations of plasma ALP and GGT at the 24 h time point compared to control sheep.

Table 8. Median (range) values for plasma concentrations of urea, creatinine and total protein, and albumin and activities of ALP, ACP and GGT in samples obtained from 6 sheep with ketoprofen induced AKI and from 6 control sheep.

Variable	Group	0 h	1 h	2 h	4 h	6 h	8 h	24 h
Urea (mmol/L)	Ketoprofen	5.3 (3.9–6.9)	5.2 (4.1–7.0)	5.8 (4.5–7.6)†	7.2 (5.6-8.8)*†	8.8 (6.6-9.6)*†	9.8 (7.1-10.4)*†	20.6 (14.0–22.3)*†
	Control	4.7 (3.5–5.2)	5.3 (3.8–6.3)	4.9 (3.6–6.1)	4.6 (3.7–5.7)	4.1 (3.4–5.7)	3.4 (2.8–5.0)	4.1 (2.6–7.2)
Creatinine (µmol/L)	Ketoprofen	86 (80–106)	88 (79–105)	89 (82–109)	120 (100–140)*†	151 (131–191)*†	184 (150–208)*†	390 (131–414)*†
	Control	92 (81–113)	96 (80–110)	89 (79–106)	91 (74–101)	90 (71–102)	88 (74–105)	90 (71–101)
Albumin (g/L)	Ketoprofen	31.6 (30.8–32.6)	31.5 (31.0-32.8)	30.1 (28.0-31.1)*†	29.6 (28.6-30.4)*†	29.4 (28.7-31.5)*†	28.6 (27.6-31.2)*†	26.4 (23.5–31.9)*†
	Control	31.8 (30.2–33.9)	31.9 (30.3-33.1)	31.6 (30.7-32.4)	31.6 (30.9–32.8)	32.6 (30.1–34.4)	31.8 (30.9–32.8)	32.0 (30.8–33.6)
Total protein (g/L)	Ketoprofen	64.8 (61.0–68.7)	64.9 (61.0-66.5)†	62.1 (59.3-63.1)*†	60.1 (56.4-60.1)*†	60.5 (54.7-66.0)*†	57.6 (52.0-65.2)*†	51.8 (42.9–68.1)*†
	Control	67.0 (63.0–69.5)	66.2 (63.4-68.7)	66.4 (64.8–68.2)	66.5 (63.7–67.9)	67.1 (63.5–72.3)	67.0 (64.8–68.9)	66.3 (60.9–68.3)
ALP (U/L)	Ketoprofen	229 (175–296)	196 (159–296)	202 (164–296)	198 (131-288)†	205 (136–302)	186 (128–312)	121 (72–312)*†
	Control	202 (184–337)	212 (199–259)	201 (189–252)	186 (167–276)	203 (187–266)	201 (169–300)	200 (171–300)
ACP (U/L)	Ketoprofen	3.8 (3.4–4.1)	NM	NM	NM	NM	9.3 (5.0–11.2)*†	7.5 (4.3–10.2)*†
	Control	3.7 (3.5–4.0)	NM	NM	NM	NM	3.8 (3.6–4.1)	3.8 (3.4–3.9)
GGT (U/L)	Ketoprofen	46 (37–69)	44 (36–67)	42 (35–65)†	43 (35–62)†	43 (36–64)†	40 (34–59)†	36 (30–48)*†
	Control	46 (41–52)	45 (40–49)	47 (41–50)	47 (41–51)	48 (42–50)	46 (42–50)	46 (41–49)

*Within a time point within a variable, value differs significantly ($P < 0.05$) from the value for the control sheep.
†Within a row, value differs significantly ($P < 0.05$) from the value for baseline.

8.1.4 FINDINGS IN KIDNEY TISSUE

Immunostaining of the sheep kidney tissue with anti-calbindin D28k and anti-CD1d (II), and with anti-C1q, anti-C3c, anti-C3d, anti-C4c, anti-C5, anti-C9 and anti-factor H (III) revealed differences in staining patterns between the treated group and the controls (Figure 4). In control sheep kidney tissue, no signal of calbindin D28k, CD1d, C1q or factor H was detected, as the basement membranes of blood vessels, epithelial cells in tubuli and Bowman's capsule in glomeruli were positively stained for C3c. In addition, the tubulointerstitium in the medulla showed positive staining for C3d and distal convoluted tubules and proximal tubule epithelia in the cortex for C4c in control sheep. C5 and C9 were also present in proximal tubule epithelial cells in the medulla.

After ketoprofen overdose in sheep, the deposition of C3c was more intense compared to control sheep in the epithelial cells of proximal tubules in the medulla and in the tubular lumina. Similarly to control sheep, the kidney of the ketoprofen-exposed sheep showed positive staining for C4c in distal convoluted tubules and proximal tubule epithelia in the cortex. In addition, positive staining was also seen in proximal tubules in the medulla, where C4c localized in epithelial cells and associated with cellular debris in the tubular lumina. C3d showed positive staining in the proximal tubule epithelial cells and in tubular lumen in the medulla in all ketoprofen-overdosed sheep, and also in the distal convoluted tubules in the cortex in two ketoprofen-exposed sheep. C1q was found in the proximal tubules in the medulla and in the tubular lumina after ketoprofen overdose. C5 and C9 showed positive staining in the distal convoluted tubules and proximal tubule epithelia, and in the tubular lumina, intensifying from the cortex to the medulla. Factor H showed strong positive staining in the proximal tubules in the inner medulla in all affected sheep. Two sheep with ketoprofen overdose also showed positive staining for factor H in proximal tubules in the outer medulla and distal convoluted tubules in the cortex area.

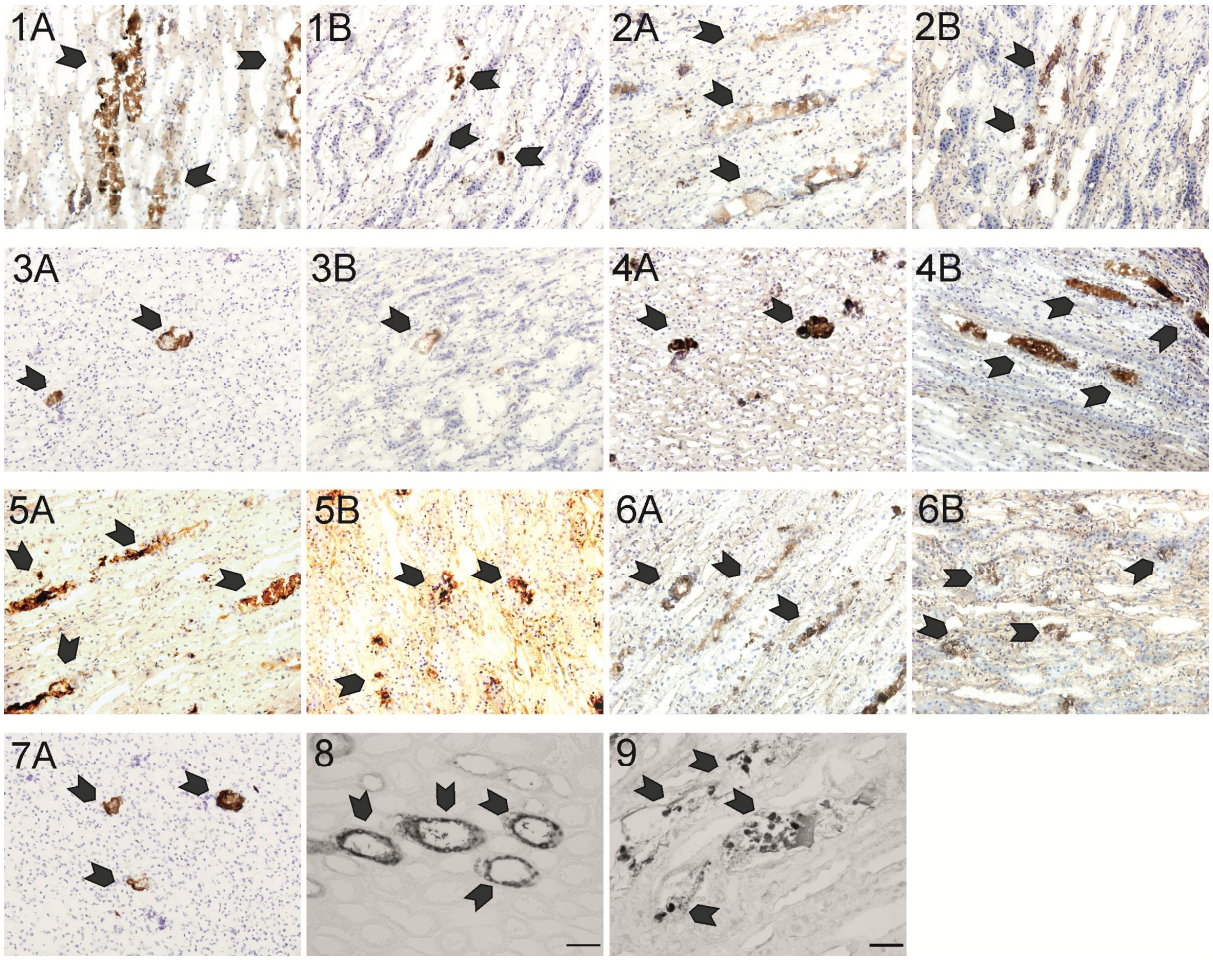

Figure 4. Immunostaining of kidney tissue of sheep with ketoprofen overdose-induced AKI: 1A) cortex C1q; 1B) medulla C1q; 2A) cortex C3c; 2B) medulla c3c; 3A) cortex C3d 3B) medulla C3d; 4A) cortex C4c; 4B) medulla C4c; 5A) cortex C5; 5B) medulla C5; 6A) cortex C9; 6B) medulla C9 7A) cortex fH; 8) medulla calbindin D28k; 9) medulla CD1d. The arrowheads indicate the positively stained antigens in the cortex and medulla in sheep after NSAID overdose. Figures 1A–7 are counterstained with hematoxylin–eosin. The original magnifications are 200X in Figures 1A–7A and 400X in Figures 8–9.

8.2 ENVENOMATION IN DOGS

8.2.1 ROUTINE CLINICAL CHEMISTRY ANALYSIS, CLINICAL GRADATION AND KIDNEY FUNCTION SCORE

Table 9 summarizes the findings in blood from dogs bitten by the common adder and treated in the Veterinary Teaching Hospital of the University of Helsinki. The variation in all variables was wide, the range being outside of both minimum and maximum reference values on the day of admission.

Four of the 34 dogs were assessed to suffer from minor envenomation (grade 1) and 19 dogs from moderate envenomation (grade 2). Severe envenomation was encountered in 9 dogs (grade 3). The impact of envenomation on the kidneys was assessed and 12 of the 32 dogs did not have any specific findings in their routine clinical chemistry analysis (grade 1), 15 showed mild deterioration (grade 2) and five of the dogs showed severe deterioration (grade 3) suggestive of kidney impairment.

Table 9. Mean values together with standard deviation of plasma concentrations of total protein, albumin, creatinine and urea from dogs bitten by the common adder on the day of admission (day 1), and the highest value measured or the sample taken on the day after admission (day 2).

Variable	**Day 1** **Mean ± SD** **(min–max)**	**Day 2** **Mean ± SD** **(min–max)**	**Reference range**
Total protein	49.0 ± 11.5 g/L (35–78 g/L)	45.7 ± 9.2 g/L (32–65 g/L)	58–77 g/L
Albumin	28.4 ± 6.7 g/L (18.9–49 g/L)	24.3 ± 6.1 g/L (12–36.5 g/L)	30–41 g/L
Creatinine	116.3 ± 114.9 µmol/L (27–626 µmol/L)	100.8 ± 106.4 µmol/L (40–564 µmol/L)	57–116 µmol/L
Urea	9.1 ± 8.6 mmol/L (2.1–39.6 mmol/L)	7.8 ± 9.8 mmol/L (2.2–44.8 mmol/L)	2.4–8.8 mmol/L

8.2.2 FINDINGS IN URINE

In 2D-DIGE analysis (IV), seven proteins were significantly upregulated in the urine of dogs bitten by *Vipera berus berus* compared to the control group. Five of them were identified by PMF: β2MG, AAT, albumin, Fetuin-B and SOD1 (Table 10).

Table 10. Proteins expressed differently ($P < 0.05$) in the urine of dog bitten by *Vipera berus berus* compared to controls, and detected by 2D-DIGE coupled with LC-MS/MS (IV).

Identified protein	**Accession number**	**Theoretical pI/MW**	**Matched peptides**	**Sequence coverage %**	**MASCOT score**	**Ratio**
AAT	gi|121583756	5.58/46.3	24	16.2	558	3.05
Albumin	gi| 3319897	5.52/68.6	36	41.6	1043	2.73
B2MG	XP_535458	5.92/14.2	22	28.6	307	8.42
Fetuin-B	gi|74003556	5.64/42.3	8	37.1	478	4.4
SOD1	Q8WNN6	5.69/15.6	9	21.6	235	2.66

Urinary b2MG, RBP4 and calbindin D28k were measured by ELISA analysis. The b2MG:creatinine ratio (mean ± standard deviation [min–max]) of nine dogs bitten by the common adder was 0.25 ± 0.22 µg/mg (0.04–0.78 µg/mg), while in the control dogs the index was 0.050 ± 0.007 µg/mg (0.04–0.05 µg/mg). The RBP4:creatinine ratio (median [min-max]) of dogs bitten by common adder was 0.0018 (0.00–0.03) and that of control dogs 0.0028 (0.00–0.4). Urinary calbindin D28k was measured successfully only in six dogs bitten by *Vipera berus berus*, but in all control dogs. The urinary calbindin D28k ratio to creatinine (mean and CI) was 0.0065 (0.003–0.015) in dogs bitten by the common adder and 0.0045 (0.0009–0.020) in healthy control dogs. The urinary creatinine concentration was (geometric mean [CI]) 15 514.2 µmol/L (3023.3–34 785.4 µmol/L) in control dogs and 2412.3 µmol/L (556.9–5542.7 µmol/L) in dogs bitten by *Vipera berus berus*. Urinary enzyme activity indices and protein:creatinine ratios differed significantly ($P < 0.001$) between controls and cases (Table 11).

Table 11. Urine creatinine ratios of measured variables in dogs bitten by the common adder and healthy controls.

	U-GGT:creatinine ratio*	**U-ALP:creatinine ratio***	**U-Protein: creatinine ratio***
Control	0.003 (0.0007–0.0068)	0.001 (0.0004–0.0032)	0.001 (0.0002–0.0022)
Case	0.007 (0.002–0.017)	0.01 (0.003–0.024)	0.009 (0.003–0.023)
P	<0.001	<0.001	<0.001

*Geometric mean and confidence interval

Positive correlations were recorded between urinary creatinine ratios of ALP, GT, and total protein. The envenomation grade correlated with urinary creatinine ratios of ALP ($R = 0.642$, $P < 0.001$), GT ($R = 0.619$, $P < 0.001$) and total protein ($R = 0.669$, $P < 0.001$). The kidney function score correlated with the urinary creatinine ratio of ALP ($R = 0.603$, $P < 0.001$).

9 DISCUSSION

9.1 BLOOD VARIABLES

The plasma concentration of creatinine was significantly higher by 4 h after the administration of a ketoprofen overdose in sheep in study I, suggesting reduced kidney function. However, the creatinine plasma concentration did not exceed the RIFLE criteria for AKI in humans (Westhuyzen et al., 2003; Venkataraman and Kellum, 2007; Endre et al., 2008) until 8 hours after the initial insult to the kidneys. As urine enzyme:creatinine ratios already showed a statistically significant increase two hours after the initial insult to the kidneys, it is suggested that evaluation only based on plasma creatinine is not a sensitive marker in the early diagnosis of AKI in sheep exposed to an overdose of ketoprofen. The administered dose of ketoprofen was 10 times higher than the therapeutic dose for cattle (3 mg/kg). As a result, the sheep developed AKI in a relatively short period of time (<24 h). As the localization of COX isoforms in the sheep kidney is not known, it is unclear how susceptible sheep kidney tissue is to ketoprofen. Therefore, it is unclear whether the given dose could mimic clinical settings for an accidental overdose of NSAID in clinical veterinary patients. In study V, in dogs bitten by *Vipera berus berus*, the plasma creatinine concentration at the time of admission was above the reference range in eight (25%) dogs and below the reference range in seven (21.9%) dogs. It was also noticed that the serum creatinine concentration in dogs bitten by *Vipera berus berus* was not necessarily associated with the severity of the symptoms or measured urinary analytes. Comparing the sample taken on admission to the hospital with the highest value measured or the sample taken the day after admission, the plasma creatinine concentration either increased or decreased in dogs bitten by the common adder. The same changes in serum creatinine were seen in dogs bitten by snakes having venom known to consist of cytotoxic or neurotoxic ingredients (Hrovat et al., 2013). The evaluation of kidney function based on merely serum creatinine levels appears to be ineffective after common adder envenomation in dogs.

In study I, both plasma albumin and total protein concentrations were already significantly lower in sheep with ketoprofen-induced AKI than control sheep two

hours after exposure, and the concentrations continued the decline until end of the study. In dogs bitten by the common adder, the concentrations of albumin and total protein were substantially lower than the reference values. The decline in both analytes seen in sheep with ketoprofen overdose-induced AKI as well as dogs bitten by the common adder, associated with proteinuria, suggests increased excretion. Plasma leakage from the blood circulation to the tissues, the administered fluid therapy, or reduced albumin translation in the liver may also have contributed to the decreased plasma concentration of albumin.

9.2 URINARY ENZYMES

Studies I and V both suggested that urinary enzyme:creatinine ratios were sensitive indicators of kidney impairment. Brush border enzymes, such as ALP and GGT, are typically released into the urine during less severe injuries, compared with release of lysosomal or cytosolic enzymes (D'Amico and Bassi, 2003; Westhuyzen et al., 2003). In study I, the increases in urinary activities of the lysosomal enzymes NAG and ACP and the cytosolic LDH suggested that relatively severe injury to tubular cells had already occurred within two hours after the administration of ketoprofen. An increase in the urinary activities of ALP and GGT followed after that within four hours after administration. The concurrent decrease in plasma ALP and GGT activities detected after administration of ketoprofen suggested that some of these urinary enzyme activities may have originated from plasma. In study V, the urinary ratios of GGT:creatinine and ALP:creatinine differed significantly between affected and control dogs, supporting the findings in study I. According to a systematic review by Coca et al. (2008), evaluation of urinary GGT activity performs well in the early diagnosis of AKI in humans. The association between urinary ratios of GGT, ALP and protein-to-creatinine in domestic animals with toxic insult to the kidneys (I, V) supports the use of these parameters when evaluating possible kidney impairment. The increased urinary creatinine ratios of NAG, ACP and LDH (I) suggested tubular cell rupture and the release of these enzymes to urine.

9.3 URINARY PROTEINS

In proteomic studies on urine after toxic insult to the kidneys by NSAID overdose or common adder envenomation (II, IV and unpublished data), several proteins were found to be upregulated. The majority of identified proteins had a low molecular weight, suggesting that both NSAIDs and common adder venom cause tubular injury. A disadvantage in gel-based proteomics is that very basic, low-abundance or hydrophobic proteins are difficult to observe. In addition, developing proteinuria contains large amount of albumin, which may hide some of the emerging low-abundance proteins. To overcome this, urine samples were pretreated with a bead-based library of combinatorial peptide ligands (unpublished data) (ProteoMiner, Bio-Rad) as described earlier (Candiano et al., 2012). As the venom of the common adder contains several ingredients that have the capacity to degrade extracellular matrix components, it is possible that proteinuria after envenomation was at least partly caused by glomerulus impairment. From the identified low-molecular-weight proteins, RBP4, β2MG and AAT are well known markers of kidney impairment in both humans and animals (Raila et al., 2000; Bianchi et al., 2001; van Hoek et al., 2008; Maddens et al., 2010; Metzger et al., 2010).

9.3.1 COMPLEMENT COMPONENTS

Several antigens were localized in the tubular epithelium and lumen in immunohistochemical studies on kidney tissue (II, III) after ketoprofen-induced AKI. Histological examination (I) revealed ATI in sheep administered an overdose of ketoprofen. Further examination with antibodies against C1q, C3c, C4c, C5, C9, and factor H placed the expression of these antigens mainly at the site of ATI. The differential expression between control and AKI sheep for all antigens indicated that these proteins either directly contribute to kidney destruction or are a secondary manifestation of it. AKI with different preliminary causes may share common features, and this is well recorded in ischemic, toxic and sepsis-induced AKI, where hypoxia plays a role (Heyman et al., 2012).

Complement components are believed to have a leading role in proteinuria-associated tubulointerstitial injury (Sheerin and Sacks, 2002).

Complement activation is associated with a large variety of renal disorders with proteinuria in both humans and animals, such as glomerulonephritis, renal infarction, ischemia-reperfusion injury and IgA nephropathy (Meri et al., 1992; Väkevä et al., 1995; Farrar et al., 2006; Jang and Rabb 2008; Onda et al., 2011). The complement system consists of soluble and membrane-bound proteins, and it contributes strongly in the mediation of inflammation, tissue clearance and protection against infection (Müller-Eberhard, 1988; Walport, 2001). Some of the circulating C3 originates from the tubular compartment in the kidney, but the majority of complement C3 in plasma is synthesized in the liver (Tang et al., 1999). The excretion of complement components C3 and C4 was increased after NSAID-induced AKI (unpublished data). The MASCOT search identified C3 and C4 as their complete forms, but the measured MWs were lower than theoretically calculated for both C3 and C4 (<72 kDa). The increase in excretion may be a result of gain of expression of C3 and C4 in tubular epithelial cells, together with enzymatic cleavage products of C3 and C4. Complement regulatory proteins such as factor H, membrane cofactor protein (MCP) and decay accelerating factor (DAF) inhibit further activation of the C cascade in the normal kidney (Pangburn et al., 1981; Ichida et al., 1994; Timmerman et al., 1996; Gerritsima et al., 1997; Yamada et al., 2004). The main soluble inhibitor of the alternative pathway, factor H, binds to the cell surface and together with factor I breaks down C3b to an inactive form (Zipfel et al., 1999). Proximal tubular cells, which have very low expression of membrane-bound complement regulators such as DAF and MCP, bind factor H, yet excess plasma proteins can prevent the interaction (Buelli et al., 2009).

In the case of renal inflammation, or in proteinuric diseases, the synthesis of C3 in the kidney increases (Sacks et al., 1993; Montinaro et al., 2000). Activated C proteins cluster in the epithelium of proximal tubules after proteinuria in humans (Camussi et al., 1983; Biancone et al., 1994; Gerritsma et al., 1997) and rats (Eddy et al., 1989; Nomura et al., 1997). The end product of C activation is the formation of C5b-9 (membrane attack complex, MAC), which comprises one molecule each of C5b, C6, C7 and C8, and one or more molecules of C9 (Müller-Eberhard 1988). MAC adheres to the cell membrane and induces cell injury and apoptosis (Biancone et al., 1994).

The positive immunostaining of C3c, C4c, C1q, C5, C9 and factor H in kidney tissue after ketoprofen-induced AKI (III) principally occurred in the proximal tubular epithelial cells and in the tubular lumen, but also to some extent in the distal convoluted tubular epithelial cells. This finding implies that intrarenal synthesis of complement components could promote the progression of ketoprofen-induced AKI. Our study cannot confirm whether MAC was formed, but the positive immunostaining for complement components, especially C5 and C9, suggests that the MAC complex is formed and is at least partly responsible for the cell injury seen in tubular cells after NSAID overdose.

9.3.2 OTHER PROTEINS

AAT and β2MG were detected in study IV in dogs bitten by the common adder. They are both freely filtered by the glomerulus and normally reabsorbed in the tubules, and are therefore considered to be markers of tubular injury (Yanagisawa et al., 1983; Herget-Rosenthal et al., 2004). AAT is an acute-phase glycoprotein produced in the liver, and secreted into the bloodstream. It belongs to the serine protease inhibitor (SERPIN) family, contributes to the regulation of proteolysis and protects the extracellular matrix during inflammation (du Bois et al., 1991; Gettins, 2002). AAT protects kidney tissue by anti-apoptotic and anti-inflammatory routes in renal ischemic/reperfusion injury (Daemen et al., 2000). β2MG belongs to the major histocompatibility complex (MHC) class I molecules and is filtered by the renal glomerulus (Schardijn and Statius van Eps, 1987). β2MG is reabsorbed from epithelial cells of the proximal tubules by megalin- and cubilin-mediated endocytosis and is degraded to amino acids (Schardijn and Statius van Eps, 1987; Verroust et al., 2002). RBP4 is the specific carrier of vitamin A in plasma. When bound to vitamin A it forms a complex with transthyretin, increasing the molecular weight of RBP4 and preventing its loss in the kidney glomeruli (Kanai et al., 1968, Naylor et al., 1999). Released RBP4 is freely filtered by the glomerulus and reabsorbed by the proximal tubule cells (Peterson et al., 1970), and its increased concentration in urine may thus also suggest a malfunction of this process.

The other identified low-molecular-weight proteins, i.e. calbindin D28k (II), CD1d (II), fetuin-B (IV) and SOD1 (IV), participate in various biological functions

and are also all expressed in the kidney tissue (Canchis et al., 1993; Marklund et al., 1982; Takada et al., 1982; Fredericks and Bosch, 1997; Denecke et al., 2003; Brigl and Brenner, 2004). Two of these proteins were connected to NSAID overdose-induced AKI: calbindin D28k and CD1d (II). Calbindin D28k is a cytosolic vitamin D-dependent calcium-binding protein and is expressed in the distal convoluted tubule, where calcium reabsorption is regulated (Parmentier et al., 1987). The positive immunostaining of calbindin D28k in the sheep kidney tubules after ketoprofen overdose (II) supports the theory that the excretion of calbindin D28k is likely to stem from disrupted epithelial cells.

CD1d is expressed on cell surfaces and belongs to non-polymorphic major histocompatibility complex (MHC) class I-like molecules (Bilsland et al., 1991). In the kidney, the expression of CD1d is concentrated in the glomeruli and some of the tubules, and in smooth muscle surrounding blood vessels (Canchis et al., 1993). Antigen-presenting cells expressing MHC class I-like molecules such as CD1d are presented to NKT cells in the kidney, among a large variety of other tissues (Brigl and Brenner, 2004). The positive immunostaining of CD1d in sheep kidney after an overdose of ketoprofen (II) suggests that NKT cells could be present in renal tissue after toxic insult. The expression of CD1d in tubular epithelial cells and tubular lumina suggests that the excreted protein may originate from the epithelial cells. Yang et al. (2011) reported that human patients with an unknown aetiology of ATN had NKT in kidney tissue, particularly localized in the tubulointerstitial area, and the number of NKT cells varied according to the severity of ATN. Several studies involving mouse models have also connected NK and NKT cells to tubular injury (Li et al., 2007; Zhang et al., 2008; Satpute et al., 2009; Yang et al., 2011). The role of innate immunity in NSAID-induced AKI remains uncertain, although the occurrence of CD1d and complement proteins implies it.

The two other proteins found in urine, fetuin-B and SOD1, were associated with common adder envenomation (IV). Fetuin-B is a small (42.3 kDa) extracellular protein originating from the liver that inhibits cysteine-type endopeptidase activation. It belongs to the cystatin superfamily of proteases and is a paralogue to fetuin-A, sharing some of the functions (Olivier et al., 2000; Denecke et al., 2003). Some snake venoms contain endopeptidases (Tu and Toom, 1967; Bottrall et al., 2010), and the manifestation of fetuin-B in the urine

of dogs bitten by the common adder (IV) may thus indicate that common adder venom also contains endopeptidase activity. The most probable origin of excreted protein is plasma, where fetuin-B is released from the site of cell rupture.

Copper/zinc superoxide dismutase (SOD1) is a small (15.06 kDa) cytosolic enzyme belonging to a metal-containing enzyme family that catalyses the dismutation of superoxide anions and is expressed in all mammalian tissues (Marklund et al., 1982; Takada et al., 1982; Fredericks and Bosch, 1997). These enzymes act as the most important line of antioxidant defence against ROS, metabolic by-products that can lead to oxidative stress (Zelko et al., 2002; Johanson and Giulivi, 2005). Oxidative stress has been associated with snake envenomation in humans and mice (Yamasaki et al., 2008; Zengin et al., 2012; Santhos et al., 2013). Since venom of the common adder contains SV-LAOOs that promote the release of hydrogen peroxidase, it is probable that excretion of SOD1 is related to oxidative stress in envenomed dogs. The excreted SOD1 probably at least partly originates from plasma, where it is released from the site of cell rupture. It may also be leaked from disrupted kidney cells.

In studies I, II and III with the sheep model of AKI, some changes in the urinary protein profile were also detected in the control group. The urinary protein concentration and proenzyme MMP-2 activity also slightly increased in some control sheep (I), apolipoprotein A1 appeared in the urine of both groups (II) and the excretion pattern of complement proteins was identical between groups (III). These findings hint that the urethral catheter, which remained *in situ* for eight hours, may have caused local irritation in the lower urinary tract, and therefore contributed to these urinary variables.

9.3.3 FUTURE PROSPECTS

Based on the studies included in this thesis, and as suggested earlier (Decramer et al., 2008; Thongboonkerd, 2010), urine appears be a good choice of sample matrix for detecting kidney impairment. The urinary creatinine ratios of ALP, and GGT (I, IV), together with LDH, ACP and NAG (I), could be reliable variables for diagnosing kidney impairment in its early stage. The redeeming feature of these urinary enzymes is that their measurement is often conducted with a clinical

chemistry analyser, which is readily available for most clinical sites. Several of the proteins detected in sheep or dog urine after toxic insult by proteomic methods (II, V and unpublished data) warrant further investigation with clinical veterinary patients suffering from kidney impairment. The ELISA analysis of urinary β2MG in dogs bitten by the common adder and control dogs supported the 2D-DIGE results (IV). Analysis of urinary RBP4 and calbindin D28k proved to be challenging and failed to function with urine from dogs bitten by the common adder. More research with a larger set of clinical samples and a wider range of methods is needed to validate the potential AKI biomarkers reported in this thesis.

Concerning the criteria of a good biomarker of AKI (Nguyen and Devarajan, 2008), urinary enzymes together with urinary calbindin D28k and RBP4 were upregulated in the early stage of kidney impairment after toxic insult (I,II). Although most of the detected proteins suggested tubular injury in the sheep and dog studies, glomerular impairment may also been manifested. To distinguish between glomerular and tubular injury, urinary enzymes ACP, ALP, GGT, LDH and NAG (I) together with calbindin D28k, RBP4, (II), AAT, and β2MG (IV) detected with proteomic methods appear to accurately reflect tubular injury. None of the urinary proteins reported in this study could definitely distinguish renal and nonrenal injury, although urinary fetuin-B and SOD1 found in dogs bitten by the common adder may indicate nonrenal tissue destruction related to the snake venom impact. The increase in urinary activities of the lysosomal enzymes NAG and ACP, and the cytosolic LDH in sheep after toxic insult may serve as a tool for assessing the severity of tubular injury.

10 CONCLUSIONS

1. Changes in activities of several enzymes were detected, and these changes proved to be sensitive markers for kidney impairment after NSAID overdose in sheep and envenomation by *Vipera berus berus* in dogs.

2. Several new potential urinary markers, such as calbindin-D28k, CD1d, C3β, C4γ, SOD1 and Fetuin-B, were detected together with those previously reported in the literature (RBP4, β2MG and AAT) after a toxic insult resulting from NSAID or common adder venom.

3. Complement activation participates in kidney tissue destruction after an overdose of NSAID in sheep, and components of the complement system can be detected in kidney tissue and urine.

11 AKNOWLEDGEMENTS

The research presented in this thesis was carried out at the Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki. The financial support provided by the Finnish Veterinary Foundation is gratefully acknowledged.

I would warmly thank my supervisors, Outi Vainio, Marja Raekallio and Sami Junnikkala for their support and encouragement during these years. Outi and Marja, thank you for encouraging me to start this whole project and also for guiding me through the rough spots of research and scientific writing. I thank you Sami for introducing me the basics for complement system and guiding me to the treasure chest of antibodies. Thank you all for your support and advice and for creating very fruitful atmosphere for science.

The reviewers of this thesis, Joseph Bartges and Antonia Vlahou, are greatly acknowledged for providing valuable constructive comments and suggestions that improved this thesis. I warmly thank Roy Siddall who carried out the language revision in such a short notice. Aaro Miettinen is thanked for accepting the role of opponent in my dissertation.

All my co-authors are thanked for their contributions to this thesis. Satu Sankari is warmly thanked for her help. Minna Rajamäki is thanked for her help with zymograms, supervising my master thesis, and also the support she has given me through all these years. I am ever grateful for Mari Vainionpää for her contribution to evaluating clinical laboratory results, collecting samples in the Small Animal Hospital together with Suvi Kosonen and most of all, for all the discussions we have shared. I thank Heini Lahtinen for collecting samples from healthy controls and Jere Linden for his help with interpretation of immunostainings. Seppo Meri is thanked for valuable comments and quidance concerning complement system, and also for providing antibodies.

All my co-workers in Oppilasrakennus have been the factor, why I have felt at home from the day one. My “partners in crime”, Kaisa, Merja, Taina and Kirsi, for you I am truly grateful for your support, friendship and help during all the fun times we have spent in the lab. Without you many dilution factors would have stayed as mystery. Elina, Tiina, Sanni, Heini, Miiu, Laura, Suvi, Anna, Flavia and Rachel, thank you for all the discussions between heaven and earth and generally for being there. Faik Atroshi is thanked for all the support and guidance through the years. I truly have enjoyed our discussions.

Kiitän äitiäni, Eevaa, joka rientää aina hädän hetkellä avuksi, kaikesta tuesta ja positiivisesta esimerkistä, jota hän on vuosien saatossa minulle antanut. Kiitän myös appeani Kalevia kaikesta mitä näiden vuosien aikana olemme yhdessä kokeneet ja tehneet. Kiitokseni ansaitsevat myös isäni Kalevi ja anoppini Marjatta, jotka eivät ehtineet juhlia tätä saavutusta kanssani. I also thank my brothers Jukka ja Marko, and my sister-in-laws Katriina and Minna for their support.

Lee Brice said it all : « Here's to the nights we don't remember and the friends we won't forget». Life is hectic and sometimes it seems that there is not enough time for friends. Fortunately I am blessed with friends that tolerate silent periods quite well. Thank you Johanna, Laura, Satu Elina, Kirsi, Mari, Pate and Inka, Matti and Marjo, Sami and Lotta, and Miska and Saija.

The greatest thank you goes to my family. I thank my sons, Miro and Miklas, for teaching me patience, humoring me by their shenanigans (at least in the end) and giving me the opportunity to watch them grow and blossom. Last, but not least, I would like to thank my husband Miika, who has always supported me no matter what. He has helped me with my actual lab work, listened to my problems and given me reason to laugh and I am forever grateful for that.

12 REFERENCES

Abbate, M., Zoja, C., Corna, D., Capitanio, M., Beratni, T., Remuzzi, G. In progressive nephropaties, overload of tubular cells with filtered proteins translates glomerular permeability dysfunction into cellular signals of interstitial inflammation. J Am Soc Nephrol 1998: 9: 1213-1224.

Adachi, J., Kumar, C., Zhang, Y., Olsen, J. V., Mann, M. The human urinary proteome contains more than 1500 proteins, including a large portion of membrane proteins. Genome Biol 2006: 7: R80- R80.11.

Aebersold, R., Mann, M. Mass spectrometry-based proteomics. Review. Nature 2003: 422: 198-207.

Aird, S. D. Ophidian envenomation strategies and the role of purines. Review. Toxicon 2002: 40: 335-393.

Arthur, J. M., Hill, E. G., Alge, J. L., Lewis, E. C., Neely, B. A., Janech, M. G., Tumlin, J. A., Chawla, L. S., Shaw, A. D. Evaluation of 32 urine biomarkers to predict the progression of acute kidney injury after cardiac surgery. Kidney Int. 2013 Sep 4. doi: 10.1038/ki.2013.333.

Askenazi, D. J., Koralkar, R., Hundley, H. E., Montesanti, A., Parwar, P., Sonjara, S., Ambalavanan, N. Urine biomarkers predict acute kidney injury in newborns. J Pediatr 2012: 161: 270-275.

Astor, B. C., Matsushita, K., Gansevoort, R. T., van der Velde, M., Woodward, M., Levey, A. S., de Jong, P. E., Coresh, J., the chronic kidney disease prognosis consortium. Lower estimated glomerular filtration rate and higher albuminuria are associated with mortality and end-stage renal disease. A collaborative meta-analysis of kidney disease population cohorts. Kidney Int 2011: 79: 1331-1340.

Audebert, F., Sorkine, M., Bon, C. Envenoming by viper bites in France: clinical gradation and biological quantification by ELISA. Toxicon 1992: 30, 599-609.

Bakoush, O., Tencer, J., Tapia, J., Rippe, B., Torffvit, O. Higher urinary IgM excretion in type 2 diabetic nephropathy compared to type 1 diabetic nephropathy. Kidney Int 2002: 61: 203-208.

Bandara, L., Kelly, M. D., Lock, E. A., Kennedy, S. A potential biomarker of kidney damage identified by proteomics: preliminary findings. Biomarkers 2003: 8: 272-286.

Batuman, V., Verroust, P. J., Navar, G. L., Kaysen, J. H., Goda, F. O., Cambell, W. C., Simon, E. Pontillon, F., Lyles, M., Bruno, J., Hammond, T. G. Myeloma light chains are ligands for cubilin (gp280). Am J Physiol 1998: 275: F246-F254.

Bazzi, C., Petrini, C., Rizza, V., Arrigo, G., Beltrame, A., D'Amico, G. Characterization of proteinuria in primary glomerulonephritides. SDS-PAGE patterns: clinical significance and prognostic value of low molecular weight ("tubular") proteins. Am J Kidney Dis 1997: 1: 27-35.

Bazzi, C, Petrini, C., Rizza, V., Arrigo, G., Napodano, P., Paparella, M., D'Amigo, G. Urinary N-acetyl-beta-glucosaminidase excretion is a marker of tubular cell dysfunction and a predictor of outcome in primary glomerulonephritis. Nephrol Dial Transplant 2002: 11: 1890-1896.

Behrend, E., Grauer, G. F., Mani, I., Groman, R. P., Salman, M. D., Greco, D. S. Hospital-acquired acute renal failure in dogs: 29 cases (1983-1992). J Am Vet Med Assoc 1996: 208: 537-541.

Bellei, E., Cuoghi, A., Monari, E., Bergamini, S., Fantoni, L. I., Zappaterra, M., Guerzoni, S., Bazzocchi, A., Tomasi, A., Pini, L. A. Proteomic analysis of urine in medication-overuse headache patients: possible relation with renal damages. J Headache Pain 2012: 13: 45-52.

Bennett, W. M., Henrich, W: L., Stoff, J. S. The renal effects of nonsteroidal anti-inflammatory drugs: summary and recommendations. Am J Kidney Dis 1996: 28(1): S56-62.

Bennett MR, Ravipati N, Ross G, Nguyen MT, Hirsch R, Beekman RH, Rovner L, Devarajan P. Using proteomics to identify preprocedural risk factors for contrast induced nephropathy. Proteomics Clin Appl 2008: 2(7-8): 1058-1064.

Bianchi, C.; Donadio, C.; Tramonti, G.; Consani, C.; Lorusso, P.; Rossi, G. Reappraisal of serum β2-microglobulin as marker of GFR. Ren Fail 2001: 23: 419-429.

Biancone, L., David, S., Della, P. V., Montrucchio, G., Cambi, V., Camussi, G. Alternative pathway activation of complement by cultured human proximal tubular epithelial cells. Kidney Int 1994: 45: 451-460.

Bilsland, C.A.G., Milstein, C. The Identification of the Beta-2-Microglobulin Binding Antigen Encoded by the Human Cd1d Gene. Eur J Immunol 1991: 21, 71-78.

Bohle, A:, Backensen-Haen, S:, von Gise, H. Significance of tubulointerstitial changes in the renal cortex for the excretory function and concentration ability of the kidney. Am J Nephrol 1987: 7: 421-433.

Brigl, M., Brenner, M. CD1: Antigen presentation and T cell function. Annu Rev Immunol 2004: 22, 817-890.

Brown, S. A., Finco, D. R:, Boudinot, F. D:, Wright, J., Taver, S. L., Cooper, T. Evaluation of a single injection method, using iohexol, for estimating glomerular filtration rate in cats and dogs. Am J Vet Res 1996: 57: 105-110.

Brune, K., Hinz, B. Selective cyclooxygenase-2 inhibitors: similarities and differences. Scand J Rheumat 2004: 33: 1-6.

Bröker ME, Lalmahomed ZS, Roest HP, van Huizen NA, Dekker LJ, Calame W, Verhoef C, Ijzermans JN, Luider TM. Collagen peptides in urine: a new promising biomarker for the detection of colorectal liver metastases. PLoS One. 2013 Aug 16;8(8):e70918. doi: 10.1371/journal.pone.0070918.

Buelli, S., Abbate, M., Morigi, M., Moioli, D., Zanchi, C., Noris, M., Zoja, C., Pusey, C. D., Zipfel, P. F., Remuzzi, G. Protein load impairs factor H binding promoting complement-dependent dysfunction of proximal tubular cells. Kidney Int 2009: 75: 1050-1059.

Burdmann, E. A.; Woronik, V.; Prado, E. B. A.; Abdulkader, R. C.; Saldanha, L. B.; Barretto, O. C. O.; Marcondes, M. Snakebite-induced acute kidney failure: an experimental model. Am J Med Hyg 1993: 48: 82-88.

Burke, J. E., Dennis, E. A. Phospolipase A_2 structure/function, mechanism, and signaling. J Lipid Res 2009: 50(Suppl): S237-242.

Burne, M. J., Osicka, T. M., Comper, W. D. Fractional clearance of high molecular weight proteins in conscious rats using continuous infusion method. Kidney Int 1999: 55: 261-270.

Câmpean, V., Theilig, F., Paliege, A., Breyer, M., Bachmann, S. Key enzymes for renal prostaglandin synthesis: site-specific expression in rodent kidney (rat, mouse). Am J Physiol Renal Physiol. 2003: 285: F19-32.

Camussi G, Rotunno M, Segoloni G, Brentjens JR, Andres GA. In vitro alternative pathway activation of complement by the brush border of proximal tubules of normal rat kidney. J Immunol 1982: 128: 1659–1663.

Camussi, G., Tetta, C., Mazzucco, G., Vercellone, A. The brush border of proximal tubules of normal human kidney activates the alternative pathway of the complement system in vitro. The J Immunol 1983: 420: 321-324.

Camussi G, Stratta P, Mazzucco G, Gaido M, Tetta C, Castello R, Rotunno M, Vercellone A: In vivo localization of C3 on the brush border of proximal tubules of kidneys from nephritic patients. Clin Nephrol 1985: 23: 134–141.

Canchis, P.W., Bhan, A.K., Landau, S.B., Yang, L., Balk, S.P., Blumberg, R.S. Tissue distribution of the non-polymorphic major histocompatibility complex class I-like molecule, CD1d. Immunology 1993: 80: 561-565.

Candiano, G., Santucci, L., Bruschi, M., Petretto, A., D'Ambrosio, C., Scaloni, A., Righetti, P. G., Ghiggeri, G. M. "Cheek-to-cheek" urinary proteome profiling via combinatorial peptide ligand libraries: A novel, unexpected elution system. J Proteomics 2012: 75: 796-805.

Capone, M. L., Tacconelli, S., Sciulli, M. G., Grana, M., Ricciotti, E., Minuz, P., Di Gregorio, P., Merciaro, G., Patrono, C., Patrignani, P. Clinical pharmacology of platelet, monocyte, and vascular cyclooxygenase inhibition by naproxen and low-dose aspirin in healthy subjects. Circulation. 2004: 109: 1468-1471.

Carone, F. A., Peterson, D. R., Oparil, S:, Pullman, T. N. Renal tubular transport and catabolism of proteins and peptides. Kidney Int 1979: 16: 271-278.

Christensen, E. I., Gburek, J. Protein reabsorption in renal proximal tubule-function and dysfunction in kidney pathophysiology. Pediatr Nephrol 2004: 19: 714-721.

Coca SG, Yalavarthy R, Concato J, et al. Biomarkers for the diagnosis and risk stratification of acute kidney injury: a systematic review. Kidney Int 2008: 73: 1008–1016.

Cockcroft, D. W., Gault, M. H. Prediction of creatinine clearance from serum creatinine. Nephron 1976, 16: 31-41.

Cui, S., Verroust, P. J., Moestrup, S. K., Christensen, E. I. Megalin/gp330 mediates uptake of albumin in renal proximal tubules. Am J Physiol 1996: 271: F900-F907.

Daemen, MA.; Heemskerk, VH.; van't Veer, C.; Denecker, G.; Wofs, TG.; Vandenabeele, P.; Buurman, WA. Functional protection by acute phase proteins α1-acid glycoprotein and α1-antitrypsin against ischemia/reperfusion injury by preventing apoptosis and inflammation. Circulation 2000: 102: 1420-1426.

Daltry, J. C., Ponnudurai, G., Shin, C. K., Tan, NH., Thorpe, R. S., Wüster, W. Electrophoretic profiles and biological activities: intreaspecific varation in the venom of the Malayan pit viper (*Calloselasma rhodostoma*). Toxicon 1996: 34: 67-79.

D'Amigo, G., Bazzi, C. Pathophysiology of proteinuria. Kidney Int 2003: 63: 809-825.

de Castro, I.; Burdmann, E. de A.; Seguro, A. C.; Yu, L. *Bothrops* venom induces direct renal tubular injury: role for lipid peroxidation and prevention by antivenom. Toxicon 2004, 43: 833-839.

Decramer, S., Gonzalez de Peredo, A., Breuil, B., Mischak, H., Monsarrat, B., Bascands, J. L., Schanstra, J. P. Urine in clinical proteomics. Review. Mol Cell Proteomics 2008: 7: 1850-1862.

Denecke, B.; Gräber, S.; Schäfer, C.; Heiss, A.; Wöltle, M.; Jahnen-Dechent, W. Tissue distribution and activity testing suggest a similar but not identical function of Fetuin-B and fetuin-A. Biochem J 2003: 376: 135-145.

De Roodt, AR.; Lago, NR.; Stock, RP. Myotoxicity and nephrotoxicity by *Micrurus* venoms in experimental envenomation. Toxicon 2012, 59:356-364.

deSequera, P., Albalate, M., Hernandez, J., Vazquez, A., Abad, J., Ramiro, E., Guerrero, M. F., Caramelo, C., Casado, S., Ortiz, A. Acute renal failure due to sulphadiazine crystalluria in AIDS patients. Postgrad Med J 1996: 72: 557-558.

Dinchuk, J. E., Car, B. D., Focht, R. J., Johnston, J. J., Jaffee, B. D., Covington, M. B., Contel, N. R., Eng, V. M., Collins, R. J., Czernial, P. M. Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. Nature 1995: 378: 406-409.

Donadelli, R., Zanchi, C., Morigi, M., Buelli, S., Batani, C., Tomasoni, S., Corna, D., Rottoli, D., Benigni, A., Abbate, M., Remuzzi, G., Zoja, C. Protein overload indices fractalkine upregulation in proximal tubular cells through nuclear factor κB- and p38 mitogen-activated protein kinase-dependent pathways. J Am Soc Nephrol 2003: 14: 2436-2446.

du Bois, RM.; Bernaudin, JF.; Paakko, P.; Hubbard, R.; Takahashi, H.; Ferrans, V.; Crystal, RG. Human neutrophils express the alpha 1-antitrypsin gene and produce alpha 1-antitrypsin. Blood 1991: 77: 2724-2730.

Durnwald, C., Mercer, B. A prospective comparison of total protein/creatinine ratio versus 24-hour urine protein in women with suspected preeclampsia. Am J Obstet Gynecol 2003: 189: 848–852.

Eddy, A. A., McCulloch, L., Adams, J., Liu, E. Interstitial nephritis induced by protein-overload proteinuria. Am J Pathol 1989: 135(4): 719-733.

Eddy, A. A., Giachelli, C. M. Renal expression of genes that promote interstitial inflammation and fibrosis in rats with protein-overload proteinuria. Kidney Int 1995: 47: 1546-1557.

Endre ZH, Westhuyzen J. Early detection of acute kidney injury: emerging new biomarkers. Nephrol (Carlton) 2008: 13: 91–98.

Escalante, T., Shannon, J., Moura-da-Silva, A. M., Gutierrez, J. M., Fox, J. W. Novel insights into capillary vessel basement membrane damage by snake venom hemorrhagic metalloproteinases: a biochemical and immunohistochemical study. Arch Biochem Biophys 2006: 455: 144-153.

Farrar, CA., Zhou, W., Lin, T. Sacks, SH. Local extravascular pool of C3 is a determinant of postischemic acute renal failure. FASEB J 2006: 20: 217-226.

Fortenberry, J. D., Pade, M. L., Goldstein, S. L. Acute kidney injury in children: An update on diagnosis and treatment. Pediatr Clin N Am 2013: 60: 669-688.

Fox, J. W., Serrano, S. M. Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. Toxicon, 2005: 45: 969-985.

Fredericks, WM.; Bosch, KS. Localization of superoxide dismutase activity in rat tissues. Free Radical Bio Med 1997: 22: 241-248.

Freeman WE, Couto CG, Gray TL. Serum creatinine concentrations in retired racing Greyhounds. Vet Clin Pathol. 2003: 32: 40-42.

Fridén, V., Oveland, E., Tenstad, O., Ebefors, K., Nyström, J., Nilsson, U. A., Haraldsson, B. The glomerular endothelial cell coat is essential for glomerular filtration. Kidney Int 2011: 79: 1322-1330.

Funk, C. D. Prostaglandins and leukotrienes: advances in eicosanoid biology. Review. Science 2001: 294: 1871-1875.

Georgieva, D., Risch, M., Kardas, A., Buck, F., von Bergen, M., Betzel, C. Comparative analysis of the venom proteomes of *Vipera ammondytes ammondytes* and *Vipera ammondytes meridionalis*. J Proteome Res 2008, 7: 866-886.

Gerritsima, JSJ., Gerritsen, AF., De Ley, M., van Es, LA,, Daha, MR. Interferon-γ induces biosynthesis of complement components C2, C4 and factor H by human proximal tubular epithelial cells. Cytokine 1997: 9: 276-283.

Gettins, P. G. W. Serpin structure, mechanism and function. Chem Rev 2002: 102: 4751-4803.

Girish, K. S., Jagadeesha, D. K., Rajeev, K. B., Kemparaju, K. Snake venom hyaluronidase: an evidence for isoforms and extracellular matrix degradation. Mol Cell Biochem 2002: 240: 105-110.

Gleadhill. A., Michell, A. R. Evaluation of iohexol as a marker for the clinical measurement of glomerular filtration rate in dogs. Res Vet Sci 1996: 60: 117-121.

Good DM, Zürbig P, Argilés A, Bauer HW, Behrens G, Coon JJ, Dakna M, Decramer S, Delles C, Dominiczak AF, Ehrich JH, Eitner F, Fliser D, Frommberger M, Ganser A, Girolami MA, Golovko I, Gwinner W, Haubitz M, Herget-Rosenthal S, Jankowski J, Jahn H, Jerums G, Julian BA, Kellmann M, Kliem V, Kolch W, Krolewski AS, Luppi M, Massy Z, Melter M, Neusüss C, Novak J, Peter K, Rossing K, Rupprecht H, Schanstra JP, Schiffer E, Stolzenburg JU, Tarnow L, Theodorescu D, Thongboonkerd V, Vanholder R, Weissinger EM, Mischak H, Schmitt-Kopplin P. Naturally occurring human urinary peptides for use in diagnosis of chronic kidney disease. Mol Cell Proteomics 2010: 11: 2424-2437.

Goodison, S., Rosser, C., Urquidi, V. Urinary proteomic profiling for diagnostic bladder cancer biomarkers. Expert Rev Proteomic 2009: 6: 507-514.

Grönlund, J., Vuori, A.; Nieminen, S. Adder bites. A report of 68 cases. Scand J Surg 2003, 92: 171-174.

Gubensek, F., Sket, D.; Turk, V.; Lebez, D. Fractionation of *Vipera ammondytes* venom and seasonal variation of its composition. Toxicon 1974: 12: 167-168.

Hall, I. E., Coca, S. G, Perazella, M. A., Eko, U. U., Luciano, R. L., Peter, P. R., Han, W. K., Parikh, C. R. Risk of poor outcomes with novel and traditional biomarkers at clinical AKI diagnosis. J Am Soc Nephrol 2011: 6: 2740-2749.

Haller, M., Muller, W., Binder, H., Estelberger, W., Arnold, P. Single-injection inulin clearance – a simple method for measuring glomerular filtration rate in dogs. Res Vet Sci 1998: 64: 151-156.

Han, W. K., Waikar, S. S., Johnson, A., Betensky, R. A., Dent, C. L., Devarajan, P., Bonventre, J. V. Urinary biomarkers in the early diagnosis of acute kidney injury. Kidney Int 2008: 73: 863-869.

Handa, S. P. Drug-induced acute interstitial nephritis: report of 10 cases. CMAJ 1986: 135: 1278-1281.

Haraldsson, B., Sörensson, J. Why do we not all have proteinuria? An update of our current understanding of the glomerular barrier. News Physiol Sci 2004: 19: 7-10.

Harris RC, McKanna JA, Akai Y, Jacobson HR, Dubois RN, Breyer MD. Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. J Clin Invest. 1994: 94: 2504-2510.

Hei, Z. Q., Li, X. Y., Shen, N., Pang, H. Y., Zhou, S. L., Guan, J. Q. Prognostic values of serum cystatin C and beta2 microglobulin, urinary beta2 microglobulin and N-acetyl-beta-D-glucosaminidase in early acute renal failure after liver transplantation. Chin Med J 2008: 121:1251-6.

Heiene R, Moe L, Mølmen G. Calculation of urinary enzyme excretion, with renal structure and function in dogs with pyometra. Res Vet Sci 2001: 70: 129-37.

Heinrikson, R. L., Krueger, E. T., Keim, P. S. Amino acid sequence of phospolipase A_2 –alpha from the venom of Crotalus adamanteus. A new classification of phospolipases A_2 based upon structural determinants. J Biol Chem 1977: 252: 4913-4921.

Herget-Rosenthal, S.; Poppen, D.; Hüsing, J.; Marggraf, G.; Pietruck, F.; Jakob, H-G.; Philipp, T.; Kribben, A. Prognostic value of tubular proteinuria and enzymuria in nonoligouric acute tubular necrosis. Clin Chem 2004: 50: 552-558.

Heyman, S. N., Evans, R. G., Rosen, S:, Rosenberger, C. Cellular adaptive changes in AKI: mitigating renal hypoxic injury. Nephrol Dial Transplant 2012: 27: 1721-1728.

Hickey, E. J., Raje, R. R., Reid, V. E., Gross, S. M., Ray, S. D. Diclofenac induced in vivo nephrotoxicity may involve oxidative stress-mediated massive genomic DNA fragmentation and apoptotic cell death. Free Radic Biol Med 2001 15: 31: 139-152.

Hilppö M. Some haematological and clinical-chemical parameters of sight hounds (Afghan hound, saluki and whippet). Nord Vet Med. 1986: 38: 148-55.

Hinchcliff KW, Olson J, Crusberg C, Kenyon J, Long R, Royle W, Weber W, Burr J. Serum biochemical changes in dogs competing in a long-distance sled race. J Am Vet Med Assoc. 1993: 202: 401-405.

Hinton, L. E., McLoughlin, M. A., Johnson, S.E., Weisbrode, S. E. Spontanous gastroduodenal perforation in 16 dogs and seven cats (1982-1999). J Am Anim Hosp Assoc 2002: 38: 176-187.

Ho, J., Lucy, M., Krokhin, O., Hayglass, K., Pascoe, E., Darroch, G., Rush, D., Nickerson, P., Rigatto, C., Reslerova, M. Mass spectrometry-based proteomic analysis of urine in acute kidney injury following cardiopulmonary bypass: a nested case-control study. Am J Kidney Dis 2009: 53: 584-595.

Hoste, EAJ., Clermont, G., Kertsen, A., Venkataraman, R., Angus, DC., De Bacquer, D., Kellum, JA. RIFLE criteria for acute kidney injury are associated with hospital mortality in critically ill patients: a cohort analysis. Critical care 2006, 10: R73.

Hreinsson, J. P., Kalaitzakis, E., Gudmunsson, S., Björnsson, E. S: Upper gastrointestinal bleeding: incidence, etiology and outcomes in a population-based setting. Scand J Gastroenterol 2013: 48: 439-447.

Hrovat, A., Schoeman, J. P., de Laat, B., Meyer, E., Smets, P., Goddard, A:, Nagel, S., Daminet, S. Evaluation of snake envenomation-induced renal dysfunction in dogs using early urinary biomarkers of nephrotoxicity. Vet J 2013, pii: S1090-0233(13)00314-6. doi: 10.1016/j.tvjl.2013.06.030.

Ichida, S., Yuzawa, Y., Okada, H., Yoshioka, K., Matsuo, S. Localization of the complement regulatory proteins in the normal human kidney. Kidney Int 1994: 46: 89-96.

Ichimura, T., Bonventre, J. V., Bailly, V., Wei, H., Hession, C. A., Cate, R. L., Sanicola, M. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion

molecule containing a novel immunoglobulin domain, is up-regulated in renal cell after injury. J Biol Chem 1998: 273: 4135-4142.

Jang, HR, Rabb, H. The innate immune response in ischemic acute kidney injury. Cl Immunol 2008: 130: 41-50.

Jayanthi, G. P., Gowda, T. V. Geographical variation in India in the composition and lethal potency of Russell's viper (Vipera russelli) venom, Toxicon 1988, 26 , 257-264.

Jeansson, M., Haraldsson, B. Glomerular size and charge selectivity in the mouse after exposure to glucosaminoglycan-degrading enzymes. J Am Soc Nephrol 2003: 14: 1756-1765.

Jensen AL, Wenck A, Koch J, Poulsen JS. Comparison of results of haematological and clinical chemical analyses of blood samples obtained from the cephalic and external jugular veins in dogs. Res Vet Sci. 1994: 56: 24-29.

Joachim, G. R., Cameron, S., Schwartz, M., Becker, E. L. Selectivity of protein excretion in patients with the nephrotic syndrome. J Clin Invest 1964: 43: 2332-2346.

Johanson, F.; Giulivi, C. Superoxide dismutases and their impact upon human health. Mol Aspects Med 2005: 26. 340-352.

Karlson-Stieber, C; Salmonson, H and Persson, H. A nationwide study of *Vipera berus* bites during one year – Epidemiology and morbidity of 231 cases. Clin Toxicol 2006, 44:24-30.

Kanai, M., Raz, A., Goodman, D.S. Retinol-binding protein: the transport protein for vitamin A in human plasma. J Clin Invest 1968: 47, 2025-2044.

Kerjaschki, D. Dysfunctions of cell biological mechanisms of visceral epithelial cell (podocytes) in glomerular diseases. Kidney Int 1994: 45: 300-313.

Khan TN, Sinniah R: Role of complement in renal tubular damage. Histopathology 1995: 26: 351–356.

Khan, K. N. M., Venturini, C. M., Bunch, R. T., Brassard, J. A., Koki, A. T., Morris, D. L., Trump, B. E., Maziasz, T. J., Alden, C. L. Interspecies differences in renal localization of cyclooxygenase isoforms: implications in nonsteroidal anti-inflammatory drug-related nephrotoxicity. Toxicol pathol 1998: 26: 612-620.

Khan, K. N. M., Paulson, S. K., Verburg, K. M., Lefkowith, J. B., Maziasz, T. J. Pharmacology of cyclooxyhenase-2 inhibition in the kidney. Kidney Int 2002: 61: 1210-1219.

Kinsey, G., Li, L., Okusa, M. Inflammation in acute kidney injury. Nephron Exp Nephrol 2008: 109: e102-e107.

Kovacevic, L., Bernstein, J., Valentini, R. P., Imam, A., Gupta, N., Mattoo, T. K. Renal papillary necrosis induced by naproxen. Pediatr Nephrol 2003: 18: 826-829.

Kömhoff, M., Gröne, H. J., Klein, T., Seyberth, H. W., Nüsing, R. M. Localization of cyclooxygenase-1 and -2 in adult and fetal human kidney: implication for renal function. Am J Physiol 1997: 272: F460-F468.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970: 227: 680–685.

Lameire, N., Vanholder, R. Pathophysiologic features and prevention of human and experimental acute tubular necrosis. J Am Soc Nephrol 2000: 12(Suppl 17): S20-23.

Laurie, G. W., Leblond, C. P., Inoue, S., Martin, G. R., Chung, A. Fine structure of the glomerular basement membrane and immunolocalization of five basement membrane components to the lamina densa (basal lamina) and its extensions in both glomeruli and tubules of the rat kidney. Am J Anat 1984: 169: 463-481.

Layton, D., Souverein, P. C., Heerdink, E. R., Shakir, A. W., Egberts, A. C. G. Evaluation of risk profiles for gastrointestinal and cardiovascular adverse effects in nonselective NSAID and COX-2 inhibitor users. Drug Safety 2008: 31: 143-158.

Lee, YJ., Hu, YY., Lin, YS., Chang, CT., Lin, FY., Wong, ML., Kuo-Shuan, H., Hsu, WL. Urine neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute canine kidney injury. Vet Res 2012: 8: 248-256.

Lemley, K. V., Lafayette, R. A., Safai, M., Derby, G., Blouch, K., Squarer, A., Myers, B. D. Podocytopenia and disease severity in IgA nephropathy. Kidney Int 2002: 61: 1475-1485.

Lervik, J. B.; Lilliehöök, I.; Frendin, J. HM. Clinical and biochemical changes in 53 Swedish dogs bitten by the European adder – *Vipera berus*. Acta Vet Scand 2010, 52:26-37.

Levey, A. S:, Bosch, J. P., Breyer Lewis, J., Greene, T., Rogers, N., Roth, D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Ann Intern Med 1999, 130: 461-470.

Li, Z. Y., Yu, T. F., Lian, E. C. Purification and characterization of L-amino acid oxidase from king cobra (Ophiophagus hannah) venom and its effects on human platelet aggregation. Toxicon 1994: 32: 1349-1358.

Li, L., Huang, L., Sung, S., Lobo, P. I., Brown, M. G., Gregg, R. K., Engelhard, V. H., Okusa, M. D. NKT cell activation mediates neutrophil IFN-γ production and renal ischemia-reperfusion injury. J Immunol 2007: 178: 5899-5911.

Li, P. K. T., Burdmann, E. A., Mehta, R. L. Acute kidney injury: Global health alert. Transplantation 2013: 5: 653-657.

Little, D., Jones, S. L., Blikslager, A. T. Cyclooxygenase (COX) inhibitors and the intestine. J Vet Intern Med: 2007: 21:3: 367-377.

Maddens, B.E.J., Daminet, S., Demeyere, K., Demon, D., Smets, P., Meyer,E. Validation of immunoassays for the candidate renal markers C-reactive protein, immunoglobulin G, thromboxane B2 and retinol binding protein in canine urine. Vet Immunol Immunopathol: 2010: 134: 259-264.

Maddens B, Ghesquière B, Vanholder R, Demon D, Vanmassenhove J, Gevaert K, Meyer E. Chitinase-like proteins are candidate biomarkers for sepsis-induced acute kidney injury. Mol Cell Proteomics 2012: 11: M111.013094. doi: 10.1074/mcp.M111.013094.

Mandal and Bhattacharyya. Ability of a small, basic protein isolated from Russell's viper venom (Daboia russelli russelli) to induce renal tubular necrosis in mice. Toxicon 2007: 50: 236-250.

Marcussen, N. Atubular glomeruli and the structural basis for chronic renal failure. Lab Invest 1992: 66: 265-284.

Marklund, SL.; Westman, G.; lundgren, E.; Roos, G. Copper- and zinc-containing superoxide dismutase, mangane-containing superoxide dismutase, catalase and glutathione peroxidase in normal and neoplastic human cell lines and normal human tissues. Cancer Res 1982: 42: 1955-1961.

Master, F. and Kornalik, R. W. A comparative examination of yellow and white venoms of Vipera ammodytes. Toxicon 1964: 2: 109-111.

Maunsbach, A. B. Absorption of I125-labelled homologuos albumin by rat kidney tubular cells. A study of microperfused single proximal tubules by electron microscopic autoradiography and histochemistry. J Ultrastruct Res 1966: 15: 197-241.

McDuffie, J. E., Sablad, M., Ma, J., Snook, S. Urinary parameters predictive of cisplatin-induced acute renal injury in dogs. Cytokine 2010: 52: 156-162.

Meri, S., Koistinen, V., Miettinen, A., Törnroth, T., Seppälä, I. J. Activation of the alternative pathway of complement by monoclonal lambda light chains in membranoproliferative glomerulonephritis. J Exp Med 1992: 175: 939-950.

Metzger, J.; Kirch, T.; Schiffer, E.; Ulger, P.; Mentes, E.; Brand, K.; Weissinger, EM.; Haubitz, M.; Mischack, H.; Herget-Rosenthal, S. Urinary excretion of twenty peptides forms an early and accurate diagnostic pattern of acute kidney injury. Kidney Int 2010: 78: 1252-1262.

Miller, S. B. Prostaglandins in health and disease: an overview. Semin Arthritis Rheum: 2006: 36: 37-49.

Mingatto, F. E., Santos, A. C., Uyemura, S. A., Jordani, M. C., Curti, C. In vitro interaction of nonsteroidal anti-inflammatory drugs on oxidative phosphorylation of rat kidney mitochondria: respiration and ATP synthesis. Arch Biochem Biophys 1996 15: 334: 303-308.

Mischak, H., Allmaier, G., Apweiler, R., Attwood, T., Baumann, M., Benigni, A., Bennett, S. E., Bischoff, R., Bongcam-Rudloff, E., Capasso, G., Coon, J. J., D'Haese, P., Dominiczak, A. F., Dakna, M., Dihazi, H., Ehrich, J. H., Fernandez-Llama, P., Fliser, D., Frokiaer, .J, Garin, J., Girolami, M., Hancock, W. S., Haubitz, M., Hochstrasser, D., Holman, R. R., Ioannidis, J. P., Jankowski, J., Julian, B. A., Klein, J. B., Kolch, W., Luider, T., Massy, Z., Mattes, W. B., Molina, F., Monsarrat, B., Novak, J., Peter, K., Rossing, P., Sánchez-Carbayo, M., Schanstra, J. P., Semmes, O. J., Spasovski, G., Theodorescu, D., Thongboonkerd, V., Vanholder, R., Veenstra, T. D., Weissinger, E., Yamamoto, T., Vlahou, A. Recommendations for biomarker identification and qualification in clinical proteomics. Sci Transl Med 2010: 2: 46: 1-6.

Mischak, H., Ioannidis, J. P., Argiles, A., Attwood, T. K., Bongcam-Rudloff, E., Broenstrup, M., Charonis, A., Chrousos, G. P., Delles, C., Dominiczak, A., Dylag, T., Ehrich, J., Egido, J., Findeisen, P., Jankowski, J., Johnson, R. W., Julien, B. A., Lankisch, T., Leung, H. Y., Maahs, D., Magni, F., Manns, M. P., Manolis, E., Mayer, G., Navis, G., Novak, J., Ortiz, A., Persson, F., Peter, K., Riese, H. H., Rossing, P., Sattar, N., Spasovski, G., Thongboonkerd, V., Vanholder, R.,

Schanstra, J. P., Vlahou, A. Implementation of proteomic biomarkers: making it work. Eur J Clin Invest 2012: 42: 1027-1036.

Monti, P., Benchekroun, G., Berlato, D., Archer, J. Initial evaluation of canine urinary cystatin C as a marker of renal tubular function. J Small Anim Pract 2012: 53: 254-259.

Montinaro, V., Lopez, A., Monno, R., Cappiello, V., Manno, C., Gesualdo, L., Schena, F P. Renal C3 synthesis in idiopathic membranous nephropathy: correlation to urinary C5b-9 excretion. Kidney Int 2000: 57: 137-146.

Morita, Y., Ikeguchi, H., Nakamura, J., Hotta, N., Yazawa, Y., Matsuo, S. Complement activation products in the urine from proteinuric patients. J Am Soc Nephrol 2000: 11: 700–707.

Mukherjee, D., Nissen, S. E., Topol, E.J. Risk of cardiovascular events associated with selective COX-2 inhibitors. JAMA 2001: 286: 954-959.

Müller-Eberhard, HJ. Molecular organization and function of the complement system. Annu Rev Biochem 1988: 57: 321-347.

Nabity, M. B., Lees, G. E., Dangott, L. J., Cianciolo, R., Suchodolski, J. S., Steiner, J. M. Proteomic analysis of urine from male dogs during early stages of tubulointerstitial injury in a canine model of progressive glomerular disease. Vet Clin Pathol 2011: 40: 222-236.

Nabity, M. B., Lees, G. E., Cianciolo, R., Boggess, M. M., Steiner, J. M., Suchodolski, J. S. Urinary biomarkers of renal disease in dogs with X-linked hereditary nephropathy. J Vet Intern Med 2012: 26: 282-293.

Nangaku, M. Mechanisms of tubulointerstitial injury in the kidney: Final common pathway to end-stage renal failure. Review. Intern Med: 2004: 43: 9-17.

Naylor, H.M., Newcomer, M.E. The structure of human retinol-binding protein (RBP) with its carrier protein transthyretin reveals an interaction with the carboxy terminus of RBP. Biochemistry 1999: 38, 2647-2653.

Neurath, H. Evolution of proteolytic enzymes. Science 1984: 224: 350-357.

Nguyen, M. T., Devarajan, P. Biomarkers for the early detection of acute kidney injury. Pediatr Nephrol 2008: 23: 2151-2157.

Nomura, A., Morita, Y., Maruyama, S., Hotta, N., Nadai, M., Wang, L., Hasegawa, T., Matsuo, S. Role of complement in acute tubulointerstitial injury of rats with aminonucleoside nephrosis. Am J Pathol 1997: 151: 539-547.

Oates, J. A., FitzGerald G. A., Branch, R. A., Jackson, E. K., Knapp, H. R., Roberts II, L. J. Clinical implications of prostaglandin and thromboxane A_2 formation. N Engl J Med 1988: 319: 689-698, 761-767.

O'Farrel, P. H. High resolution two-dimensional electrophoresis of proteins. J Biol Chem 1975: 250(: 4007-4021.

Ohse, T., Inagi, R., Tanaka, T., Ota, T., Miyata, T., Kojima, I., Ingelfinger, J. R., Ogawa, S., Fuhita, T., Nangaku, M. Albumin induces endoplasmic reticulum stress and apoptosis in renal proximal tubular cells. Kidney Int 2006: 70: 1447-1455.

Olivier, E.; Soyry, E.; Ruminy, P.; Husson, A.; Parmentier, F.; Daveau, M.; Salier, J-P. Fetuin-B, a second member of the fetuin family in mammals. Biochem. J 2000: 350: 589-597.

Onda, K., Ohsawa, I., Ohi, H., Tamano, M., Mano, S., Wakabayashi, M., Toki, A., Horikoshi, S., Fujita, T., Tomino, Y. Excretion of complement proteins and its activation marker C5b-9 in IgA nephropathy in relation to renal function. BMC Nephrology 2011: 12: 64-72.

Pallet, N., Bouvier, N., Legendre, C., Gilleron,J., Codogno, P., Beaune, P., Thervet, E., Anglicheau, D. Autophagy protects renal tubular cells against cyclosporine toxicity. Autophagy 2008: 4: 783-791.

Pangburn MK., Schreuber, RD., Müller-Ebernhard, HJ. Formation of the initial C3 convertase of the alternative complement pathway. J Exp Med 1981: 154: 856-867.

Park, M. Y., Choi, S. J., Kim, J. K., Hwang, S. D., Lee, Y. W. Urinary cystatin C levels as a diagnostic and prognostic biomarker in patients with acute kidney injury. Nephr 2013: 18: 256-262.

Parmentier, M., Ghysens, M., Rypens, F., Lawson, D.E.M., Pasteels, J.L., Pochet, R. Calbindin in vertebrate classes: Immunohistochemical localization and Western blot analysis. Gen Comp Endocr 1987: 65: 399-407.

Patterson, M. K., Castellsague, J., Walker, A. M. Hospitalization for peptic ulcer and bleeding in users of selective COX-2 inhibitors and nonselective NSAIDs with special reference to celecobix. Pharmacoepidemiol Drug Saf. 2008: 17: 982-988.

Peng, J., Gygi, S. P. Proteomics: the move to mixtures. J Mass Spectrom 2001: 36: 1083-1091.

Pennington, S. R., Wilkins, M. R., Hochstrasser, D. F., Dunn, M. J. Proteome analysis: from protein characterization to biological function. Trends Cell Biol 1997: 7: 168-173.

Pelander, L., Ljungvall, I., Häggström, J. Myocardial cell damage in 24 dogs bitten by the common European viper (*Vipera berus*). Vet Rec 2010: 166: 687-690.

Perazella, M. A. Renal vulnerability to drug toxicity. Review. Clin J Am Soc Nephrol 2009: 4: 1275-1283.

Peterson, P.A., Berggård, I. Isolation and properties of a human retinol-transporting protein. J Biol Chem 1970: 246, 25-33.

Petite, J. Viper bites: treat or ignore? Swiss Med Wkly 2005, 135: 618-625.

Petrak, J., Ivanek, R., Toman, O., Cmelja, R., Cmejlova, J., Vyoral, D., Zivny, J., Vulpe, C. D. Déjà vu in proteomics. A hit parade of repeatedly identified differentially expressed proteins. Proteomics 2008: 8: 1744-1749.

Pieper, R., Gatlin, C.L., McGrath, A.M., Makusky, A.J., Mondal, M., Seonarain, M., Field, E., Schatz, C.R., Estock, M.A., Ahmed, N., Anderson, N.G., Steiner, S. Characterization of the human urinary proteome: A method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1400 distinct protein spots. Proteomics. 2004: 4: 1159-74.

Pisoni R, Ruggenenti P, Remuzzi G. Drug-induced thrombotic microangiopathy: incidence, prevention and management. Drug Saf 2001: 24: 491-501.

Price, R. G. Urinary enzymes, nephrotoxicity and renal disease. Toxicol 1982: 23: 99-134.

Prendergast, B. D., George, C. F. Drug-induced rhabdomyolysis – mechanisms and management. Postgrad Med J 1993: 69: 333-336.

Raila, J., Buchholz, I., Aupperle, H., Raila, G., Schoon, H-A., Schweigert,F. J. The distribution of vitamin A and retinol-binding protein in the blood plasma, urine, liver and kidneys of carnivores. Vet Res. 2000: 31: 541-551.

Raila, J., Kohn, B., Brunnberg, L., Schweigert, F. J. Effects of chronic renal disease on the transport of vitamin A in plasma and urine of dogs. Am J Vet Res 2003: 64: 874-849.

Rao, P.V., Lu, X., Standley, M., Pattee, P., Neelima, G., Girisesh, G., Dakshinamurthy, K. V., Roberts, C. T. Jr., Nagalla, S. R. Proteomic identification of urinary biomarkers of diabetic nephropathy. Diabetes care 2007, 30, 629-637.

Ratcliffe, P. J.; Pukrittayakamee, S.; Ledingham, J. G. G.; Warrell, D. A. Direct nephrotoxicity of Russell's viper venom demonstrated in the isolated perfused rat kidney. Am J Med Hyg 1989, 40: 312-319.

Rautenbach GH, Joubert HF. A comparison of health parameters in two different canine populations. Part II: Chemical pathology data. J S Afr Vet Assoc. 1988: 59: 135-138.

Ray, W. A., Stein, C. M., Daugherty, J. R., Hall, K., Arbogast, P. G., Griffin, M. R. COX-2 selective non-steroidal anti-inflammatory drugs and risk of serious coronary heart disease. Lancet: 360: 1071-1073.

Reading, C.J. Incidence, pathology, and treatment of adder (*Vipera berus L*) bites in man. J Accid Emerg Med 1996, 13:346-351.

Remuzzi, G., Ruggenenti, P., Benigni, A. Understanding the nature of renal disease progression. Kidney Int 1997: 51: 2-15.

Remuzzi G., Bertani, T. Pathophysiology of progressive nephropathies. N Engl J Med 1998: 339: 1448-1456.

Rennke, H. G., Cotran, R. S., Venkatachalam, M. A. Role of molecular charge in glomerular permeability. Tracer studies with cationic ferritins. J Cell Biol 1975: 67: 638-646.

Rivers, B. J., Walter, P. A., O'Brien, T. D., King, V.L., Polzin, D. J. Evaluation of urine gamma-glutamyl transpeptidase-to-creatinine ratio as a diagnostic tool in an experimental model of aminoglycoside-induced acute renal failure in the dog. J Am Anim Hosp Assoc 1996: 32: 323-36.

Rodby, R. A., Rohde, R. D., Sharon, Z. Pohl, M A., Bain, R. P., Lewis, E. J. The urine protein to creatinine ratio as a predictor of 24-hour urine protein excretion in type 1 diabetic patients with nephropathy. Am J Kidney Dis 1995: 26: 904–909.

Rose, R. J, Bloomberg, M. S. Responses to sprint exercise in the greyhound: effects on haematology, serum biochemistry and muscle metabolites. Res Vet Sci 1989: 47: 212-218.

Ross, L. Acute kidney injury in dogs and cats. Vel Clin Small Anim 2011: 41: 1-14.

Ruggenenti, P., Mosconi, L., Sangali, F., Casiraghi, F., Gambara, V., Remuzzi, G., Remuzzi, A. Glomerular size-selectivity dysfunction in NIDDM is not ameliorated by ACE inhibition or by calcium channel blockade. Kidney Int 1999: 55: 984-994.

Russell, F. E., Beuss, F. W., Woo, M. Y. Zootoxicological properties of venom phosphodiesterase. Toxicon 1963: 1: 99-108.

Sacks, S. H., Zhou, W. D., Andrews, P. A., Hartley, B. Endogenous complement C3 synthesis in immune-complex nephritis. Lancet 1993: 342: 1273-1274.

Salmon, A. H. J., Ferguson, J. K., Burford, J. L., Gevorgyan, H., Nakano, D., Harper, S. J., Bates, D. O., Peti-Peterdi, J. Loss of the endothelial glycocalyx links albuminuria and vascular dysfunction. J Am Soc Nephrol 2012: 23: 1339-1350.

Samel, M.; Vija, H.; Rönnholm, G.; Siigur, J.; Kalkkinen, N.; Siigur, E. Isolation and characterization of an apoptotic and platelet aggregation inhibiting L-amino acid oxidase from *Vipera berus berus* (common viper) venom. Biochim Biophys Acta 2006, 1764: 707-714.

Santhos, M. S., Sundaram, M. S., Sunitha, K., Kemparaju, K., Girish, K. S. Viper venom-induced oxidative stress and activation of inflammatory cytokines: a therapeutic approach for overlooked issues of snakebite management. Inflamm Res 2013: 62: 721-731.

Satpute, S. R., Park, J., M., Jang, H. R., Agreda, P., Liu, M., Gandolfo, M. T., Racusen, L., Rabb, H. The roel of T cell repertoire/antigen-specific interactions in experimental kidney ischemia reperfusion injury. J Immunol 2009: 183: 984-992.

Sawaya, B. P., Weihprecht, H., Campbell, W. R., Lorenz, J. N., Webb, R. C., Briggs, J. P., Schnermann, J. Direct Vasoconstriction as a Possible Cause for Amphotericin B-induced Nephrotoxicity in Rats. J Clin Invest 1991: 87: 2097-2107.

Scandling, J D., Myers, B. D. Glomerular size-selectivity and microalbuminuria in early diabetic glomerular disease. Kidney Int 1992: 41: 840-846.

Schardijn, G. H. C. and Statius van Eps, L. W. Beta-2-microglobulin: Its significance in the evaluation of renal function. Kidney Int 1987: 32:635-641.

Sellers, R. S:, Senes, P. B., Khan, K. N. M. Interspecies differences in the nephrotoxic response to cyclooxygenase inhibition. Drug Chem Toxicol 2004: 27: 111-122.

Sheerin, N. S., Sacks, S. H. Leaked protein and interstitial damage in the kidney: is complement the missing link? Clin Exp Immunol 2002: 130: 1-3.

Siigur, J., Trummal, K., Tõnismägi, K., Samel, M., Siigur, E., Vija, H., Tammiste, I., Subbi, J. Use of MALDI-TOF mass spectrometry for specificity studies of biomedically important proteases. Spectroscopy 2002: 16: 161-170.

Silva, F.G. Chemical-Induced Nephropathy: A Review of the Renal Tubulointerstitial Lesions in Humans. Toxicol Pathol 2004: 32: 71-84.

Silverman LR, Khan KN. "Have you seen this?" Nonsteroidal anti-inflammatory drug-induced renal papillary necrosis in a dog. Toxicol Pathol 1999: 27: 244-245.

Singh, A., Satchell, S. C., Neal, C. R., McKenzie, J. E., Tooke, J. E., Mathietson, P. W. Glomerular endothelial glycocalyx constitutes a barrier to protein permeability. J Am Soc Nephrol 2007: 18: 2885-2893.

Sirota, J. C., Walcher, A., Faubel, S., Jani, A:, McFann, K., Devarajan, P., Davis, C. L., Edelstein, C. L. Urine IL-18, NGAL; IL-8 and serum IL-8 are biomarkers of acute kidney injury following liver transplantation. BMC Nephrol 2013: 14: Doi: 10.1186/1471-2369-14-17.

Segev, G., Kass, P. H., Francey, T. A novel clinical scoring system for outcome prediction in dogs with acute kidney injury managed by hemodialysis. J Vet Intern Med 2008: 22: 301-308.

Smets, P. M. Y., Meyer, E., Maddens, B. E. J., Duchateau, L., Daminet, S. Urinary markers in healthy young and aged dogs and dogs with chronic kidney disease. J Vet Intern Med 2010: 24: 65-72.

Snow, D. H., Harris, R. C., Stuttard, E. Changes in haematology and plasma biochemistry during maximal exercise in greyhounds. Vet Rec. 1988: 123: 487-489.

Solez, K., Morel-Maroger, L., Sraer, J. D. The morphology of “acute tubular necrosis” in man: analysis of 57 renal biopsies and a comparison with the glycerol model. Medicine 1979: 58: 362-376.

Sothern RB, Farber MS, Gruber SA. Circannual variations in baseline blood values of dogs. Chronobiol Int. 1993: 10:364-382.

Stanton, M. E., Bright, R. M. Gastroduodenal ulceration in dogs. Retrospective study of 43 cases and literature review. J Vet Intern Med 1989: 3: 238-344.

Stern, A. W., Ritchey, J. W:, Hall, B., Ketz-Riley, C. J., Genova, S. G. Nonsteroidal anti-inflammatory drug-associated renal papillary necrosis in a white-tailed deer (*Odocoileus virginianus*). J Vet Diagn Invest 2010: 22: 476-478.

Suhail, S. M., Woo, K. T., Tan, H. K., Wong, K. S. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of urinary protein in acute kidney injury. Saudi J Kidney Dis Transpl 2011: 22: 739-745.

Suntravat, M., Yusuksawad, M., Sereemaspun, A., Pérez, J. C., Nuchprayoon, I. Effect of purified Russell's viper venom-factor X activator (RVV-X) on renal hemodynamics, renal function, and coagulopathy in rats. Toxicon 2011: 58: 230-238.

Suhr, SM., Kim, DS. Identification of the snake venom substance that induces apoptosis. Biochem Bioph Res Co 1996: 224: 134-139.

Tan, N-H; Ponnudurai, G. A comparative study of the biological properties of venoms from snakes of the genus *Vipera* (True adders). Comp Biochem Physiol 1990, 96b: 683-388.

Tang, S., Zhou, W., Sheerin, NS., Vaughan, RW., Sacks, SH. Contribution of renal secreted complement C3 to the circulating pool in humans. J Immunol 1999: 162: 4336-4341.

Takada, Y.; Noguchi, T.; Okabe, T.; Kajiyama, M. Superoxide dismutase in various tissues from rabbits bearing the Vx-2 carcinoma in the maxillary sinus. Cancer Res 1982: 42: 4233-4235.

Thadhani, R., Pascual, M., Bonventre, J. V. Acute renal failure. New Engl J Med 1996:334: 1448-1460.

Therland, K. L., Stubbe, J., Thiesson, H. C., Ottosen, P. D., Walter, S., Sørensen, G. L., Skøtt, O., Jensen, B. L. Cycloxygenase-2 is expressed in vasculature of normal and ischemic adult human kidney and is colocalized with vascular prostaglandin E2 EP4 receptors. J Am Soc Nephrol 2004: 15: 1189-1198.

Thoen, ME., Kerl, ME. Characterization of acute kidney injury in hospitalized dogs and evaluation of a veterinary acute kidney injury staging system. J Vet Emerg Crit Care (San Antonio) 2011: 21: 648-657.

Thomas, M. E., Brunskill, N. G., Harris, K. P. G., Bailey, E., Pringle, J. H., Furness, P. N., Walls, J. Proteinuria induces tubular cell turnover: a potential mechanism for tubular atrophy. Kidney Int 1999: 55: 890-898.

Thongboonkerd, V. Current status of renal and urinary proteomics: ready for routine clinical application? Nephrol Dial Transpl 2010, 25, 11-16.

Timmerman, JJ., Van der Woude, FJ., Van Gijlswijk-Janssen, DJ., Verweij, CL., Van Es, LA., Daha, MR. Differential expression of complement components in human fetal and adult kidneys. Kidney Int 1996: 49: 730-740.

Tu, A. T., Toom, P. M. Hydrolysis of peptides by snake venoms of Australia and New Guinea. Aust J Exp Biol Med Sci 1967: 45: 561-567.

Tu, A. T., Hendon, R. R. Characterization of lizard venom hyaluronidase and evidence for its action as a spreading factor. Comp Biochem Physiol B 1983: 76: 377-83.

UniProt. *Felis catus* and *Canis familiaris* Havcr1 protein. Retrieved 31.10.2013 from www.uniprot.org.

Unlü M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 1997: 18: 2071-2077.

Uyemura, S. A., Santos, A. C., Mingatto, F. E., Jordani, M. C., Curti, C. Diclofenac sodium and mefenamic acid: potent inducers of the membrane permeability transition in renal cortex mitochondria. Arch Biochem Biophys 1997 15: 342: 231-235.

Vaden, S. L., Levine, J., Breitschwerdt, E. B. A retrospective case-control of acute renal failure in 99 dogs. J Vet Intern Med 1997: 11: 58-64.

Vane, J. R., Bakhle, Y. S:, Botting, R. M. Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol 1998: 38: 97-120.

Vaidya, V. S., Waikar, S. S., Ferguson, M. A., Collins, F. B., Sunderland, K., Gioules, C., Bradwin, G., Matsouaka, R., Betensky, R. A., Curhan, G. C., Bonventre, J. V. Urinary biomarkers for sensitive and specific detection of acute kidney injury in humans. Clin Transl Sci 2008: 1: 200-208.

Wallace, J. L. Nonsteroidal anti-inflammatory drugs and gastroenteropathy: the second hundred years. Gastroenterology 1997: 112: 1000-1016.

Walport MJ. Complement. First of two parts. N Engl J Med 2001: 344: 1058–1066.

Wang, Y., Chen, J., Chen, L., Tay, Y-C., Rangan, G. K., Harrism D. C. H. Induction of monocyte chemoattractant protein-1 in proximal tubule cells by urinary protein. J Am Soc Nephrol 1997: 8: 1537-1545.

van Hoek, I., Daminet, S., Notebaert, S., Janssens, I., Meyer, E. Immunoassay of urinary retinol binding protein as a putative renal marker in cats. J Immunol Methods 2008: 329: 208-213.

Varghese, S. A., Powell, T. B., Janech, M. G., Budisavljevic, R. C., Almeida, J. S., Arthur, J. M. Identification of diagnostic urinary biomarkers for acute kidney injury. J Investig Med 2010: 58: 612-620.

Venkataraman R, Kellum JA. Defining acute renal failure: the RIFLE criteria. J Intensive Care Med 2007: 22: 187–193.

Weinelt, W; Sattler, R.W and Mebs, D. Persistent paresis of the facial muscle after European adder (*Vipera berus*) bite on the forehead. Toxicon 2002: 40:1627-1629.

Verroust, PJ.: Birn, H.; Nielsen, R.; Kozyraki,R.; Christensen, EI. The tandem endocytic receptors megalin and cubulin are important proteins in renal pathology. Kidney Int 2002: 62: 745-756.

Westbrook, J. A., Yan, J. H., Wait, R., Welson, S. Y., Dunn, M. J Zooming-in on the proteome: very narrow-range immobilized pH gradients reveal more protein species and isoforms. Electrophoresis 2001: 22: 2865-2871.

Westhuyzen J, Endre ZH, Reece G, et al. Measurement of tubular enzymuria facilitates early detection of acute renal impairment in the intensive care unit. Nephrol Dial Transplant 2003; 18: 543–551.

Wilcox, C. M., Clark, D. Association of nonsteroidal anti-inflammatory drugs with putcome in upper and lower gastrointestinal bleeding. Dig Dis Sci 1997: 42: 985-989.

Wildgruber, R., Harder, A., Obermaier, C., Boguth, G., Weiss, W., Fey, S. J., Larsen, P. M., Görg, A. Towards higher resolution: two-dimensional

electrophoresis of *Saccharomyces cerevisiae* proteins using overlapping narrow immobilized pH gradients. Electrophoresis 2000: 21: 2610-2616.

Vinge, L., Lee, G. E., Nielsen, R., Kashtan, C. E., Bahr, A., Christensen, E. I. The effect of progressive glomerular disease on megalin-mediated endocytosis in the kidney. Nephrol Dial Translant 2010: 25: 2458-2467.

Visweswaran P, Massin EK, Dubose TD Jr. Mannitol-induced acute renal failure. J Am Soc Nephrol. 1997: 8: 1028-33.

von zur Muhlen C, Schiffer E, Sackmann C, Zürbig P, Neudorfer I, Zirlik A, Htun N, Iphöfer A, Jänsch L, Mischak H, Bode C, Chen YC, Peter K. Urine proteome analysis reflects atherosclerotic disease in an ApoE-/- mouse model and allows the discovery of new candidate biomarkers in mouse and human atherosclerosis. Mol Cell Proteomics. 2012: 11: M111.013847. doi: 10.1074/mcp.M111.013847.

Worwang, S., Langston, C. E. Feline acute intristic renal failure: 32 cats (1997-2004). J Am Vet Med Assoc 2008: 232: 728-732.

Väkevä, A., Meri, S., Lehto, T., Laurila, P. Activation of the terminal complement cascade in renal infarction. Kidney Int 1995: 47: 918-926.

Yukel'son, L. Y., Shkinev, A. V., Khamudkhanova, S. M., Barabanshchikova, N. A. Proteinases and phospholipases A-2 from the venom of Vipera berus berus. Chem Nat Comp 1995: 31: 396-399.

Yamada, K., Miwa, T., Liu, J., Nangaku, M., Song, WC. Critical protection from renal ischemia reperfusion injury by CD55 and CD59. J Immunol 2004: 172: 3869-3875.

Yamasaki, SC.; Villarroel, JS.; Barone, JM.; Zambotti-Villela, L.; Silveira, PF. Aminopeptidase activities, oxidative stress and renal function in *Crotalus durissus terrificus* envenomation in mice. Toxicon 2008: 52: 445-454.

Yanagisawa, H. Y. U., Forbes, M. A., Cooper, E. H., Crockson, R. A., MacLennan, I. C. M. Alpha-1-microglobulin: an indicator protein for renal tubular function. J Clin Pathol 1983: 36: 253-259.

Yang, S. H., Lee, J. P., Jang, H. R., Cha, R., Han, S. S., Jeon, U. S., Kim, D. K., Song, J., Lee, D-S., Kim, Y. S. Sulfatide-reactive natural killer T cells abrogate ischemia-reperfusion injury. J Am Soc Nephrol 2011: 22: 1305-1314.

Yard, B. A., Chorianopoulos, E., Herr, D., van der Woude, F. J. Regulation of endothelin-1 and transforming growth factor-β1 production in cultured proximal

tubular cells by albumin and heparin sulphate glycosaminoglycans. Nephrol Dial Transplant 2001: 16: 1769-1775.

Zelko, IN.; Mariani, TJ.; Folz, RJ. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2) and EC-SOD (SOD3) gene structures, evolution and expression. Free Radic Biol Med 2002: 33: 337-349.

Zengin, S., Al, B., Yarbil, P., Guzel, R., Orkmez, M., Yildirim, C., Taysi, S. An assessment of oxidant/antioxidant status in patients with snake envenomation. Emerg Med Journal 2012: doi:10.1136/emermed-2012-202013.

Zhang, Z-X., Wang, S:, Huang, X., Min, W-P., Sun, H., Liu, W., Garcia, B., Jevnikar, A. M. NK cells induce apoptosis in tubular epithelial cells and contribute to renal ischemia-reperfusion injury. J Immunol 2008: 181: 7489-7498.

Zheng, J., Yao, Y., Han, L., XIao, Y. Renal function and injury in inants and young children with congenital heart disease. Pediatr Nephrol 2013: 28: 99-104.

Zhou, H.; Pisitkun, T.; Aponte, A.; Yuen, PST.; Hoffert, JD.; Yasuda, H.; Hu, X.; Chawla, L.; Shen, R-F.; Knepper, MA.; Star, RA. Exosomal Fetuin-A identified by proteomics: A novel urinary biomarker for detecting acute kidney injury. Kidney Int 2006: 70: 1847-1857.

Zipfel, P. F., Hellwage, J., Friese, M A., Hegasy, G., Jokiranta, S. T., Meri, S. Factor H and disease: a complement regulator affects vital body functions. Mol Immunol 1999: 36: 241-248.

Zoja, C., Donadelli, R., Colleoni, S., Figliuzzi, M., Bonazzola, S., Morigi, M., Remuzzi, G. Protein overload stimulates RANTES production by proximal tubular cells depending on NF-κB activation. Kidney Int 1998. 53: 1608-1615.

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