Kidney Injury Molecule 1: 
A potential biomarker of renal injury in the cat

by

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ABSTRACT

KIDNEY INJURY MOLECULE 1: A POTENTIAL BIOMARKER OF RENAL INJURY IN THE CAT

Susan Karlyn Bland
Advisor: Dorothee Bienzle
University of Guelph, 2014

The prevalence of both acute and chronic kidney disease in animals and people is increasing. Increased serum creatinine concentration indicates a decline in renal function but is insensitive for diagnosing acute kidney injury (AKI). As such, there is need to develop sensitive biomarkers of renal injury.

At the fore in human medicine is a biomarker of acute renal tubular injury called kidney injury molecule-1 (KIM-1). KIM-1 is a cell membrane glycoprotein with an extracellular domain that is shed from proximal tubular kidney cells and detectable in urine after ischemic and toxic renal injury. KIM-1 was undetectable in normal human urine, but presence in urine correlated with immunohistochemical detection of KIM-1 in injured tubular epithelial cells. For these reasons, KIM-1 was considered a good candidate to investigate as a biomarker of kidney injury in cats.

Alignment of KIM-1 sequences from human, rat, dog, and mice indicated a high degree of identity between species and conservation of a cytoplasmic tyrosine motif. Primers based on the conserved regions were used to amplify feline KIM-1 genomic and renal cDNA sequences. PCR assays used to amplify feline KIM-1 revealed the presence of three transcript variants derived by alternative splicing with exon-intron organization.
similar to KIM-1 orthologous sequences. KIM-1 was detected by IHC in tubules of the cortex and outer stripe of the outer medulla in cats with suspected naturally occurring AKI and cats with experimental unilateral ischemia/reperfusion. The same cells also expressed aquaporin 1, confirming expression of KIM-1 in the proximal convoluted tubules. Further, KIM-1 expression correlated positively with tubular injury scores and vimentin expression in the injured proximal tubules. Available urine immunoassay to rat Kim-1 yielded positive reactions with urine from 11 cats.

In conclusion, feline KIM-1 is similar but not identical to human KIM-1. Expression is increased in cats with naturally occurring and experimental kidney injury, and KIM-1 may be detected in urine. KIM-1 is expressed predominantly in cells of the S3 segment of proximal tubules, and appears to persist during dedifferentiation and repair of injured cells. Hence, detection of KIM-1 in urine is a promising indicator of kidney injury in cats.
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Thank you to Olivier Côté and Mary Ellen Clark without whom I would have floundered in the early days of this research.

Thank you to Chad Schmiedt for kindly agreeing to supply kidney sections, without these much of the additional research would not have been possible.

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Lastly I would like to acknowledge all the cats with kidney disease I have cared for over the years, both as family and patients, which sparked the interest in and provided the impetus for this research.
DECLARATION OF WORK PERFORMED

I declare that Susan Karlyn Bland performed all work, with the exceptions listed below, under the supervision of Dr. Dorothee Bienzle and an Advisory Committee composed of Dr. Anthony Abrams-Ogg and Dr. Darren Wood.

Dr. Dorothee Bienzle assisted with tissue procurement, data analysis, microscopic review, and preparation of figures and manuscripts.

Mary Ellen Clark (Department of Pathobiology, University of Guelph) assisted with immunohistochemical staining, PCR and Western blotting.

Dr. Chad Schmiedt provided the samples from cats with experimental unilateral renal ischemia/perfusion.

Dr. Anthony Abrams-Ogg assisted with procurement of clinical cases.

Dr. Olivier Côté assisted with primer design, PCR and sequence analysis.

Dr. Josepha DeLay assisted with histopathology scoring.
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<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>Amino Acid</td>
</tr>
<tr>
<td>ADMA</td>
<td>Asymmetric dimethylarginine</td>
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<td>AKIN</td>
<td>Acute Kidney Injury Network</td>
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<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
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<td>AQP-1</td>
<td>Aquaporin</td>
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<td>ATN</td>
<td>Acute tubular necrosis</td>
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<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
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<td>CK</td>
<td>Cytokeratin</td>
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<tr>
<td>CRFK</td>
<td>Crandell Reese feline kidney cells</td>
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<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
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<tr>
<td>BUN</td>
<td>Blood urea nitrogen</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylenetriamininepentaacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMAP</td>
<td>European Medicines Agency and Pharmaceuticals</td>
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<tr>
<td>ESRD</td>
<td>End stage renal disease</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde Dehydrogenase</td>
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<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
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<tr>
<td>HMW</td>
<td>High Molecular Weight</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>IRIS</td>
<td>International renal interest society</td>
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<tr>
<td>kb</td>
<td>Kilobase</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>KD</td>
<td>Kidney disease</td>
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<tr>
<td>KIM-1</td>
<td>Kidney injury molecule</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NAG</td>
<td>N-acetyl-β-D-glucosaminidase</td>
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<tr>
<td>OSOM</td>
<td>Outer stripe of the outer medulla</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PKD</td>
<td>Polycystic kidney disease</td>
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<tr>
<td>PRA</td>
<td>Prerenal azotemia</td>
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<tr>
<td>RBP</td>
<td>Retinol binding protein</td>
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<tr>
<td>RI</td>
<td>Reference interval</td>
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<tr>
<td>RIFLE</td>
<td>Risk of renal dysfunction, Injury to the kidney, Failure of kidney function, complete Loss of kidney function and End-stage renal disease</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
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<tr>
<td>SCC</td>
<td>Serum creatinine concentration</td>
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<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>SUN</td>
<td>Serum urea concentration</td>
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<tr>
<td>TIM-1</td>
<td>T-cell immunoglobulin and mucin domain-containing protein 1</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>UIA</td>
<td>Urine immunoassay</td>
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<tr>
<td>UPC</td>
<td>Urine protein to creatinine ratio</td>
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<tr>
<td>USG</td>
<td>Urine specific gravity</td>
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<tr>
<td>VAKI</td>
<td>Veterinary Acute Kidney Injury</td>
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CHAPTER ONE

INTRODUCTION

Kidney disease is common in cats, however there are either conflicting or limited data on prevalence, lifetime risk and cause. Kidney disease (KD) is defined as the presence of functional or structural abnormalities in one or both kidneys.¹ Neither etiology nor pathological process or prognosis is implied.

The term chronic kidney disease (CKD) defines KD that is irreversible, progressive and has existed for three months or longer.¹ CKD results in clinical and laboratory changes due to reduction in renal excretory, endocrine and regulatory functions. It is believed clinical signs are not evident until the functional renal mass is reduced by 66% to 75%, thereby severely reducing glomerular filtration.¹-³

Most often, acute kidney injury (AKI) is a process affecting intrinsically normal kidneys, but AKI may also be superimposed on preexisting kidney disease and may include prerenal and postrenal azotemia.²,⁴ AKI can represent a continuum of renal insults culminating in irreversible end-stage renal failure requiring renal replacement therapy, or can be reversible in its early phase. KD, CKD and AKI are not synonymous with kidney failure, kidney insufficiency, azotemia or uremia, although all may eventually lead to failure of kidney function. The former terms have often been used interchangeably, which has resulted in misdiagnosis, misguided treatment and misinterpretation of epidemiologic data. The commonly used terminology amongst
human nephrologists, CKD, AKI and end-stage renal disease (ESRD; kidney disease requiring continuous renal replacement therapy) as well as the general term KD will be used in this thesis. If other terms are used it is because the term was used by an author to denote some form of KD that may not have been well defined.

**Chronic Kidney Disease**

In cats, CKD has variable clinical features at presentation, is often unpredictable in its course, and in most cases the etiology remains unknown. KD can be congenital, as
with progressive polycystic kidney disease (PKD), or acquired from single insults such as lily toxicity, multiple sublethal kidney insults due to drug toxicity, or other conditions. There are various definitions of CKD; the most common appears to be structural and/or functional impairment of one or both kidneys that has been present for approximately three months or longer. This definition implies the condition is irreversible and generally progressive. However, the International Renal Interest Society (IRIS) and others suggest in some animals CKD may be stable 4-8 weeks after insult or decompensate (iris-kidney.com, accessed April 2014).

The lack of a consensus definition and use of vague terminology has hampered understanding of the epidemiology of CKD in veterinary medicine. In the largest study to date, spanning a 10 year period, a survey of databases at 23 veterinary colleges in North America involving 189,371 cats, found the overall prevalence of ‘renal failure’ was 1.18%. The reported incidence of ‘renal failure’ in 1980 was four in 1,000 cats, but the incidence appeared to increase by 1990 to 16 cases per 1,000 cats, suggesting an increase in CKD over time. During that same period, the prevalence of ‘renal failure’ at the
University of Minnesota was estimated to be 3.05%, with five cases per 1,000 cats in 1980 and 45 cases per 1,000 cats in 1990. ‘Renal failure’ was found to exist in all age groups, but was more common in middle aged or older cats, with 55 percent of cats over 10 years of age affected. The reason for the apparent increase in feline CKD is unknown, but might be attributable to better recognition of KD, longer cat lifespan, owners seeking and utilizing specialized veterinary care more often and/or changes in the definition of KD over time.

In 1995, in a study assessing diseases in cats and dogs at private veterinary practices in the US found the prevalence of KD in cats to be 1.9%. A more recent study of cats presented to the University Veterinary Centre in Sydney, Australia found the estimated prevalence of kidney disease to be 5.8% in cats compared to 1.1% in dogs. It has been suggested that general practitioners sufficiently recognize CKD and do not refer such cases, which may further contribute to an underestimation of the disease when using referral hospital data. A major weakness in all these studies is the lack of consensus definition or criteria for ‘renal failure’ or KD. Nevertheless, it is evident that the frequency of CKD is higher in cats than dogs, increases with age in cats, and that CKD is progressive and irreversible.

There is an absence of well-defined recent large-scale studies of CKD in cats. Most knowledge is based on studies of small numbers of cats, and often interpretation of data was questionable. In an early study by DiBartola clinical abnormalities of CKD as reported by owners at initial physical examination were highly variable, and included nonspecific signs such as lethargy, anorexia, weight loss, polyuria and polydipsia. These
signs occurred anywhere between 1 and 548 days prior to presentation, but it is likely subtle signs existed for longer but were unrecognized by owners. In cats with complete outcome data, the time to death or euthanasia from diagnosis of CKD was 1 to 420 days with a mean of 36 days, and among those cats euthanized the most common histopathological diagnosis was “chronic tubulointerstitial nephritis of unknown cause”. But, it is interesting to note in that study 36.5% of cats had underlying non-renal primary disease (lymphoma, amyloidosis, PKD, feline infectious peritonitis and others) which likely skewed survival data. The findings illustrate the difficulty of defining the prevalence and prognosis of different types of KD.

Studies of better-defined patient populations with primary CKD show longer survival. For example, Elliot and Barber followed 80 cats with newly diagnosed CKD over a period of 4 years. Cats were allocated to one of three groups based on a combination of clinical signs and presence of azotemia: compensated KD (no clinical signs but serum creatinine concentration [SCC] above reference interval [RI]), uremic KD (clinical signs of CKD and increased SCC), and ESRD (clinical dehydration, anorexia, azotemia and failure to respond to treatment). Three cats in the compensated group were later euthanized due to ESRD; their median survival was 385 days. Another 9 of 15 cats with compensated KD were still alive with stable KD at the end of the study with a median time since diagnosis of 1,272 days. Among the cats that died in the uremic KD group, median survival was 233 days, and 3 cats were still alive at the end of the study with a median time since diagnosis of 921 days. Cats with ESRD survived for less than 21 days. SCC at presentation in the compensated and uremic KD group was not
predictive of survival time, but SCC was predictive of survival in cats with ESRD. Cats in the first two groups appeared to have stable renal function for long periods of time and to then suddenly decompensate.

Recently, IRIS introduced a well-defined staging system for cats with stable CKD in an effort to facilitate evidence-based decision making for diagnosis, treatment and prognosis.\(^1\) Cats are staged based on SCC, urine specific gravity (USG), proteinuria and the degree of hypertension, in an effort to monitor progression of CKD (iris-kidney.com; accessed April 2014). While this staging system has not been validated, it could allow comparison between groups of cats in different research studies. IRIS criteria have been applied both retrospectively and prospectively in recent studies, but most authors have modified inclusion criteria and staging, which makes direct comparisons between studies difficult, and no studies have included cats in stage I (nonazotemic) KD.\(^{13-17}\) It is evident, though, that survival time is highly correlated with IRIS stage. Cats in stage II (mild azotemia) had median survival times of 1,151 days, while those with ESRD (stage IV) had median survival of 103 days.\(^{13}\) SCC at admission was strongly associated with survival time, but findings were not directly comparable to those of Elliot and Barber\(^{12}\) since cases were stratified differently. Regardless, in both studies survival times were much better than previously determined, and some cats lived many years with KD. KD detected at an earlier stage may be associated with longer survival, likely because multiple therapeutic interventions were initiated both to ameliorate clinical signs and help prevent further kidney injury.
The general lack of inclusion of cats with IRIS stage 1 and early stage 2 KD in studies poses a problem. This was illustrated in a study by Jepson et al. (2009), where 118 healthy nonazotemic geriatric cats were monitored for one year to assess predictors of development of azotemia. All cats had SCC within the laboratory RI at admission, yet 30.5% of cats developed azotemia by the end of the study. Upon further evaluation of the data, cats that developed azotemia had initial mean SCC of 1.73 mg/dL, and cats that did not develop azotemia had mean SCC of 1.52 mg/dL. Furthermore, cats that developed azotemia had lower USG at admission than cats that developed azotemia. Hence, strict application of the IRIS scale would have placed cats with SCC of 1.73 mg/dL into either stage 1 or early stage 2, implying they already had some degree of KD. This finding illustrates that commonly employed RI for SCC are insufficiently sensitive to detect early KD. Cats with CKD typically have USG <1.035, but several authors have noted there are individual cats that have persistent azotemia and USG >1.035. It is likely that such cats have some form of CKD despite relatively concentrated urine. Early detection of KD is important as therapeutic interventions and management of risk factors will likely be of greatest benefit prior to development of clinical signs.

Other predictive factors for progression of CKD have been investigated. In several studies increased serum phosphorus level was the only clinicopathologic parameter associated with an increased risk of death. But in another study serum phosphorus was a dependent risk factor for death since it correlated with SCC. Presence of anemia and CKD reduced the median survival to 84 days in one study. Yet in another study, anemia was considered a dependent variable, since it correlated with SCC at baseline.
In Boyd’s investigation, presence of anemia did not correlate with survival or SCC. Lack of consistent impact of anemia on survival may be an effect of treatment, as specific therapy for anemia was employed in many of the cases in the latter study.

Hypertension has also been considered an adverse prognostic factor for survival in cats with KD, but again results were not conclusive. Differing definitions of hypertension and variable treatment of hypertension may have resulted in differences between studies. There are experimental data from a rodent remnant kidney model (unilateral partial renal infarction and contralateral nephrectomy) of ‘renal insufficiency’ that suggest hypertension in addition to preexisting KD may accelerate progression to renal failure.

There are conflicting interpretations on the importance of proteinuria and/or altered urine protein to creatinine (UPC) ratio in KD of cats. Either or both changes can be found in cats with KD, but it is unclear whether they result from progression of CKD or hypertension, or mark underlying KD.

A recent study of factors associated with progression of CKD illustrated the need for a consistent staging system. Chakrabarti et al. found different clinicopathologic variables predicted progression of CKD as defined by a modified IRIS scoring system. The authors found 47% of cats progressed between stages within one year. Low packed cell volume and high UPC ratio predicted progression of cats in stage 2, and high plasma phosphorus levels predicted progression in stage 3. Hypertension did not influence progression, but therapy was instituted in hypertensive cats and likely ameliorated the damaging effects of hypertension.
The cause or causes of CKD are more difficult to define than the epidemiology. In the last few years numerous associations and correlations have been made between KD and certain conditions, among them vaccination with feline herpesvirus 1, calicivirus, and panleukopenia virus-containing vaccines grown in Crandell Reese feline kidney (CRFK) cells, feline immunodeficiency virus infection, chronic urinary tract infections, increased serum fibroblast growth factor 23 concentration, shortened telomeres, breed and PKD, feline morbillivirus infection, increased excretion of urinary cytokines interleukin (IL)-8 and transforming growth factor (TGF) beta, cardiac disease, leptospirosis, cyclophosphamide therapy and even chronic degenerative joint disease. However, only a few of these studies suggested a direct causative link to CKD, and there are likely many additional causes of CKD. It is probable that any insult to the kidney results in a cascade of events leading to tubulointerstitial disease and nephron loss. Most cats with CKD and ESRD have tubulointerstitial fibrosis and inflammation, and the degree of tubulointerstitial fibrosis usually correlates with SCC. Finding marked tubulointerstitial fibrosis in CKD has remained consistent throughout many years, but likely represents a final common pathway rather than a unique causative lesion. Tubulointerstitial damage from AKI is considered to be a critical step in the progression of KD to CKD and ESRD in people, and it is possible this is also the case for CKD in cats.

It is likely that numerous factors contribute to development and progression of CKD, but without systematic investigations using clearly defined criteria and outcomes these are difficult to identify. Data concerning lifetime risk of KD and frequency of
comorbid conditions in cats are lacking, but well defined in people. In people, the development in 2002 of standard definitions, a stratification system and surveillance program to measure incidence of CKD, shifted diagnosis from ESRD requiring dialysis or renal transplant to detection of early KD.\textsuperscript{47,48} These recommendations and definitions have been validated and adopted by many countries, and have allowed estimates of the prevalence of KD by age, sex, and ethnicity, and evaluation of risk factors. As a result, it was realized that KD is a growing health concern with increasing prevalence.\textsuperscript{49,50} This realization has lead to the identification, evaluation and comparison of renal biomarkers in both chronic and acute KD, and to evaluation of treatments in a more systematic way.\textsuperscript{51,52}

In 2010, researchers at the US Centers for Disease Control determined that more than 10\% of people over 20 years of age have CKD (National Chronic Kidney Disease Fact Sheet 2010, cdc.gov, accessed April 2014). This prevalence increases to 35\% in adults with diabetes and 20\% in adults with hypertension. Other risk factors for developing CKD include cardiovascular disease, obesity, elevated cholesterol, a family history of CKD, and advancing age (largely because risk factors for kidney disease become more common with age). Except for elevated cholesterol, all of the above risk factors might apply to cats. Earlier identification of KD in cats, and systematic adoption of the definitions in the IRIS staging system for CKD, might give better insight into the actual prevalence, lifetime risk, risk factors and prognosis. Proper staging would also allow comparison of treatment regimes and might facilitate a better understanding of
disease progression. More epidemiologic information may lead to better understanding of the pathogenesis and etiology of CKD and to discovery of specific risk factors.

**Acute Kidney Injury**

AKI is infrequently identified in cats, which may be due to limitations of current diagnostic approaches or due to the subclinical nature of many kidney injuries in cats. AKI is a well-known entity in human medicine. Conceptually, AKI affects intrinsically normal kidneys resulting in rapid reduction of kidney function, which in turn may cause failure to maintain fluid, electrolyte and acid-base balance homeostasis. AKI can also be superimposed on preexisting KD and may include pre- and post-renal azotemia.\(^4\) AKI can be part of a continuum of renal insults culminating in irreversible CKD requiring renal transplant or dialysis, or may be reversible in the early phase. AKI can be categorized according to the initial site of parenchymal damage as vascular, glomerular, tubular or interstitial. Kidney biopsies are infrequently obtained in small animals; therefore, AKI is generally diagnosed by identifying an inciting cause concurrent with altered biochemical findings. Hypovolemia, exposure to nephrotoxic substances and drugs, primary renal diseases (neoplasia, infection, immune-mediated disease) and systemic diseases with renal manifestations (sepsis, diabetes, cardiac disease) may all cause AKI.\(^{53,4,54}\) It should be noted that the term acute tubular necrosis (ATN) is often used interchangeably with AKI in human medicine. However, the degree of functional renal impairment as indicated by increased SCC is frequently accompanied by relatively unremarkable histological findings, suggesting routine histology is not very sensitive for detecting the sublethal damage of AKI.\(^{55-57}\)
Clinically, AKI can be divided into three broad categories: 1) prerenal (more recently in human medicine termed “volume responsive AKI” \(^5\)), characterized by effective hemodynamic failure of the kidneys without parenchymal damage and potentially reversible; 2) intrinsic or KD involving the renal parenchyma; and 3) postrenal or acute obstruction of the lower urinary tract.\(^4,5\) Regardless of category, the hallmark of AKI is a reduction in glomerular filtration rate (GFR).

AKI can also be categorized into four pathophysiologic phases: initiation, extension, maintenance, and recovery.\(^5\)\(^7\)\(^9\)\(^6\) The *initiation phase* begins with a renal insult such as ischemia, toxin exposure, obstruction or infection. At this point early intervention is paramount to prevent further progression, but clinical signs are seldom evident and SCC is not or insufficiently increased. This phase is characterized by severe cellular depletion of adenosine 5’-triphosphate (ATP) secondary to decreased renal blood flow. The lack of ATP leads to acute structural and functional alteration in proximal renal tubule cells and disruption of filamentous actin.\(^5\)\(^9\) The extent of the changes depends on both the severity and duration of the ischemic injury. The areas most sensitive to ischemia and hypoxia are the proximal tubules and outer medullary area, which receive 90% of the renal blood flow. Sublethal injury, cell swelling, loss of brush border at the apical cell surface, loss of cellular polarization, and cellular detachment from the basement membrane are early features that may lead to necrotic or apoptotic cell death.\(^5\)\(^5\) The S3 segment or straight portion of the proximal tubule located in the outer stripe of the outer medulla (OSOM) is the region most affected by ischemia in experimental models, while the S1 and S2 segments of the proximal tubule are less severely affected and injury is more likely to be
Injury to the epithelium and endothelium also induces expression of chemokines and cytokines such as IL-1, 6, and 8, as well as tumor necrosis factor-α and monocyte chemoattractant protein-1, which in turn contribute to initiating inflammation.\textsuperscript{59,60}

In the \textit{extension phase} of AKI, lesions are perpetuated by continued hypoxia and inflammation that propagate the damage within the corticomedullary junction independent of the initial insult.\textsuperscript{59,60} OSOM endothelial damage and dysfunction likely contribute to propagation of the inflammatory response even after restoration of blood flow, resulting in continued ischemic injury and cell death. Damaged endothelial cells up-regulate intercellular adhesion molecule-1, and P- and E-selectin on their cell surface leading to enhanced leukocyte adhesion and activation, which in turn perpetuates inflammation, worsens hypoxia and causes tubular obstruction.\textsuperscript{63} At 24 hours after experimental ischemia there were significant inflammatory cell infiltrates at the corticomedullary junction, and prominent cell necrosis and apoptosis in the OSOM, whereas proximal tubular cells within the outer cortex underwent cellular repair.\textsuperscript{61}

The third, \textit{maintenance phase} of AKI is variable in duration. In this phase, a critical amount of parenchymal damage has occurred, which may not be reversible and is often characterized by need for renal replacement therapy. Epithelial tubular cells may undergo dedifferentiation, migration, and proliferation and begin the repair process.\textsuperscript{59,60} In the fourth \textit{recovery phase}, renal parenchymal damage is repaired, if possible. Re-differentiation and re-polarization of epithelial cells may result in improvement of cellular function. In this stage additional injury from hypovolemia, nephrotoxin exposure
or sepsis, can delay or interrupt renal recovery. Typically, SCC decreases, and urine concentrating ability improves. Regeneration and repair may take weeks to months, and may nevertheless result in functional loss of renal reserve despite return of SCC to within RI.\textsuperscript{4,53,57}

Until 2002, there was no universally accepted biochemical definition regarding the diagnosis or classification of ‘acute renal failure’ in humans. The definitions used were broad. Most commonly accepted was “an abrupt and sustained decrease in kidney function resulting in retention of nitrogenous (urea and creatinine) and non-nitrogenous waste products”.\textsuperscript{64} This led to estimates of 1% to 25% on the occurrence of acquired ‘acute renal failure’ in intensive care units with an associated mortality of 15% to 60%.\textsuperscript{65,66} Establishment, validation and acceptance of diagnostic scoring systems has greatly enhanced the knowledge base regarding ‘acute renal failure’ in humans. At a consensus meeting in 2002, precise definitions of ‘acute renal failure’ were generated and the RIFLE scale was introduced: Risk of renal dysfunction, Injury to the kidney, Failure of kidney function, complete Loss of kidney function and End-stage renal disease.\textsuperscript{67} The scale was based on relative increases in SCC and decreases in GFR and urine output.

The Acute Kidney Injury Network (AKIN) subsequently recommended that the two last RIFLE stages be dropped for diagnostic purposes and that Risk, Injury and Failure be changed to stage 1, 2, 3.\textsuperscript{68} The following definition of AKI was adopted: “An abrupt (within 48 hours) reduction in kidney function currently defined as an absolute increase in SCC of $\geq 0.3 \text{ mg/dL}$ ($\geq 26.4 \text{ µmol/L}$), a percentage increase in SCC of $\geq 50\%$ (1.5-fold from baseline), or a reduction in urine output (oliguria of $\leq 0.5 \text{ mL/kg/hour}$ for
more than six hours).” This definition was intended for use in the clinical context of presumed adequate fluid resuscitation, and allowed intra-individual comparisons negating the effect of age, sex, weight, body mass and ethnicity. Criteria could also be applied in patients with SCC in RI and with CKD. Subsequently, the term AKI, rather than the older term acute renal failure was adopted, reflecting the belief that kidney injury leading to loss of function is a dynamic process, which may or may not lead to renal failure and culminate in ESRD.⁶⁶,⁶⁸

Small increases in SCC were sufficient to diagnose AKI in humans as shown in 9,205 patients.⁶⁹ Even small increases in SCC were associated with increased mortality, length of hospital stay and costs.⁷⁰ Using these criteria, a population based estimate of AKI was 2,147 cases per million population per year in Scotland.⁷¹ This estimate was markedly higher than previously found in smaller studies with ESRD as outcome.⁷²,⁷³ A recent systematic review of 71,000 patients showed that the relative risk of death increased with higher AKI scores regardless of the nature of the patient population or underlying disease.⁷⁴ In a prospective multicenter observational cohort study, sepsis was considered the most common cause of AKI, accounting for 47.5% of cases.⁷⁵ It has been suggested that the incidence of AKI in people has risen over time, likely due to advanced age, co-morbidities, hospital acquired infections, use of nephrotoxic agents (nonsteroidal anti-inflammatory drugs, contrast agents, aminoglycosides, and chemotherapeutic agents), and sepsis and CKD.⁷⁶ However, it is also possible that the true incidence of AKI is now more clearly defined with the application of uniform scoring systems. Unfortunately, patients recovering from AKI are believed to be at increased risk of CKD.
and progression to ESRD.\textsuperscript{77-81} In addition, AKI is an independent risk factor for death even after recovery from other illness.\textsuperscript{82} It has been argued that studies should be interpreted cautiously since most are of an observational and retrospective nature involving patients with co-morbid diseases; therefore, causality cannot be assumed.\textsuperscript{83} However, a recent meta-analysis\textsuperscript{82}, and a review\textsuperscript{84} of studies published since the application of the scoring systems, demonstrated a clear association between AKI and subsequent development of CKD in adults and children. Furthermore, experimental models of AKI induced by sublethal proximal tubular injury have provided evidence of transition from AKI to CKD due to maladaptive repair resulting in interstitial fibrosis and capillary rarefaction.\textsuperscript{85-87} Calcium oxalate nephropathy may progress to CKD due to IL-1β produced by dendritic cells that promotes inflammation resulting in tubular remodeling.\textsuperscript{88} In obstructive ureteral nephropathy it is believed that renal nerve stimulation via norepinephrine and calcitonin gene-related peptide induces apoptosis and causes release of profibrotic factors leading to CKD.\textsuperscript{89}

Regardless of mechanism or inciting cause, it should be stressed that an episode of AKI, which might have caused irreversible loss of renal mass even if functionality was recovered, renders the patient at greater risk for development of CKD over time. Overall, establishment, validation, and acceptance of a diagnostic scoring system have greatly enhanced the knowledge base regarding AKI in humans.

Application of scoring systems and new biomarkers for AKI has called into question the long-held belief that prerenal azotemia (PRA) is not a pathological process. PRA has been defined as ‘a physiologic response to reduced effective extracellular volume,
defined by decreased volume receptor stimulation and increased adrenergic activity, and 
can accompany actual volume loss, congestive heart failure, cirrhosis, nephrosis, or 
sepsis'.

A more common and simpler definition of PRA is ‘reversal of the clinical 
features of AKI 24-72 hours after fluid replacement’. However, there is no consensus 
definition of PRA. The most common definition is based on functional reduction in GFR 
with recovery of renal function after correction of hemodynamic abnormalities, and does 
not imply structural kidney injury. For these reasons, PRA is essentially a retrospective 
diagnosis. There are no tests available to distinguish PRA from early AKI, and the two 
processes could occur concurrently. A majority of studies reported none or only mild 
nonspecific renal histological changes in patients diagnosed with septic AKI. In a recent 
study of ‘prerenal acute injury’ (defined as acute azotemia with recovery within 48 hours 
and fractional sodium excretion <1%) numerous biomarkers of AKI were increased in all 
patients. Among patients with transient azotemia there was a higher mortality rate 
compared to among patients without such increase, suggesting that PRA is not a benign 
process.

As there can be overlap or concurrent PRA and AKI, the terms ‘volume-
responsive AKI’ and ‘volume-unresponsive AKI’ were proposed by the AKIN in an 
attempt to stratify patients for future studies. Some authors have argued that the 
emphasis should be on determining the site of dysfunction in the spectrum of ‘functional 
impairment’ versus ‘structural damage’ rather than on categorizing increasing SCC 
according to renal or prerenal causes. While several authors agree with redefining PRA 
as part of the AKI spectrum, there have been no accepted clear definitions or diagnostic 
criteria.
Reviews of AKI in cats have addressed causes of clinical signs of KD but understanding of the causes of subclinical injury remains incomplete. In some cases the inciting causes of AKI in cats are known, such as when cats have been exposed to lilies, melamine or ethylene glycol, or had urethral or ureteral obstruction or pyelonephritis. But often the cause of AKI is not known. In a study of 32 cats with AKI a cause was not identified in 10 cats. Only 17 of the cats survived the episode of AKI, and 9 of these had persistent azotemia. In another study of 22 cats receiving peritoneal dialysis to resolve AKI, seven had acute exacerbation of CKD with an unknown cause of acute injury. Lack of knowledge about causes of AKI in cats was further illustrated in a study of 70 cats free of neoplasia, post renal azotemia or CKD. The authors were unable to identify a cause in >90% of cases since that information was unknown due to ‘incomplete histories or owner ignorance’. In two recent retrospective studies of 42 and 132 cats with AKI it was noted that 24% and 40%, respectively, had no known cause for AKI. In these latter studies the 30 day post-discharge survival rates were 48% and 42%. It is possible that cats with AKI had clinically silent KD due to prior episodes of sublethal or nonclinical injury, and that the resultant decreased renal reserve impaired recovery from additional insults such as transient hypotension or dehydration (which are generally considered to be causes of PRA).

In a retrospective analysis of 164 critically ill dogs, AKI was diagnosed in 24 dogs using the scoring system VAKI (Veterinary Acute Kidney Injury), which is similar to that of the human AKIN. Overall mortality was 21.3%, but in dogs that developed AKI while in hospital, the mortality rate was 54.2%. Primary diagnosis, anesthesia and length
of stay were not significantly different between survivors and non-survivors. Two other studies have evaluated the occurrence AKI in dogs. Although each used unique definitions and scoring systems, there was increased mortality in dogs that developed AKI while hospitalized.\textsuperscript{112,113} While there are no comprehensive studies of hospital-acquired AKI in cats, it is reasonable to assume it does occur and is associated with a poorer prognosis, or that it may contribute to the initiation of CKD. As yet, in cats there are no studies linking AKI to sepsis, although that link is well established in humans. Reasons may be lack of standardized definitions of sepsis and AKI in cats and dogs, lack of recruitment of sufficient cases for analysis, and termination of critical illness by euthanasia prior to definitive diagnosis.

Precise definitions of AKI are lacking in veterinary medicine, and the term AKI is neither widely accepted nor clearly understood. The staging system proposed by Cowgill and Langston\textsuperscript{4} and the “Guideline Recommendations for Grading of AKI in Dogs and Cats (2013)” (iris-kidney.com, accessed April 2014), are similar to the human AKI scoring system, but have not yet been widely adopted or validated. The guidelines are intended to facilitate recognition, classification, functional stratification, and therapeutic decision-making in AKI. They can be applied to animals that are non-azotemic but at risk for AKI due to other illness. The guidelines also include intra-individual comparisons of SCC, thereby enabling more sensitive detection of AKI, and inclusion of animals with acute exacerbation of CKD. Recently, a model of AKI consisting of experimental bilateral ischemia was proposed in cats, however, the regime resulted in high mortality and extensive ATN, rendering it unsuitable to evaluate recovery from AKI.\textsuperscript{114}
Biomarkers in Feline Kidney Disease

The high incidence of KD in both humans and animals has resulted in a search for better indicators of kidney injury. Most studies in animals rely on SCC and serum urea concentration (SUN) for diagnosis of AKI or CKD due to ease of determination, yet both these measurements are only crude indicators of renal function as they lack specificity, sensitivity and prognostic value. Creatinine is generally produced at a constant rate, is not protein-bound and is freely filtered by the glomerulus. However, extra-renal factors that can influence SCC include meat diet, relative muscle mass, drug therapy, intense physical exercise, and hydration. Influences on urea concentration include hepatic function, circulating blood volume, protein catabolism (fever, starvation, corticosteroid administration, infection), dietary protein concentration, gastrointestinal hemorrhage, and drug therapy.

Arguably, a more sensitive and specific method of detecting decreased but steady-state kidney function is GFR. In humans, equations estimating GFR based on SCC are commonly used. These equations take into account pre-analytic variables such as age, race, sex and weight. Similar equations have not been evaluated in veterinary medicine.

The widespread use of GFR as a measure of renal function in cats has been hampered by the lack of ‘normal’ GFR references intervals for different methods and by poor reproducibility. Robust studies correlating different methods with the gold standard of inulin clearance are for the most part lacking. Urinary clearance of creatinine...
during exogenous creatinine clearance testing is comparable to inulin clearance, but is not easy to perform in general practice. In addition, there is lack of information regarding pre-analytic variables that may affect GFR such as age, anesthesia, body type, sex or breed. Lastly, GFR is most commonly standardized to body weight expressed in kg, however, renal clearance may not be linearly related to body weight.

In a series of studies of various protocols to measure GFR in both normal, aged euthyroid and hyperthyroid cats, it was found that techniques differed significantly and that values obtained with one technique were not directly comparable to those obtained with other techniques. Van Hoek et al. found determination of GFR by scintigraphy prior to treatment of hyperthyroid cats valuable for detecting subclinical KD, and for predicting which cats would have clinically important declines in renal function following treatment for hyperthyroidism. McClellan et al. compared radioactive $^{99m}$-Tc-diethylenetriamininepentaacetic acid (DTPA) to inulin clearance in healthy cats receiving intravenous fluids, furosemide or mannitol. In all instances $^{99m}$-TcDTPA as compared to inulin clearance underestimated GFR. The recommendation was that scintigraphy may be used to monitor response to treatment but should not be used for exact measurement of GFR. However, although scintigraphy is among the least invasive methods for measuring GFR, since neither anesthesia nor potentially nephrotoxic iodinated contrast media is required, and it may be modified for single kidney studies, it is not readily available.

The use of a single plasma iohexol clearance test has recently been investigated in dogs and cats with and without CKD. Single iohexol clearance significantly correlated to
three-sample iohexol clearance and was useful in detecting decreased GFR.\textsuperscript{128-131} The results were similar to those in a prior study of partially nephrectomized cats designed to mimic CKD.\textsuperscript{132} However, there are several limitations to the use of iohexol: first, laboratory measurement of iohexol is not readily available; second, it has not been evaluated in cats with naturally occurring KD; and third, the administration of contrast material to a patient with pre-existing KD may be contraindicated. Contrast-induced nephropathy is a well-recognized entity in human medicine that occurred in as many as 1 of 6 intensive care patients.\textsuperscript{133,134}

Also, while every classification scheme of AKI in humans relies on a threshold value or change in SCC, no single SCC corresponds to a given GFR across all patients.\textsuperscript{119} Therefore, it is the change in SCC that is clinically and physiologically useful in determining the presence of AKI.\textsuperscript{67} SCC varies little on a day-to-day basis in both humans\textsuperscript{135,136} and cats.\textsuperscript{137} As such, intra-individual (within-subject) biological variation is smaller than inter-individual (between-subject) biological variation, and probably currently the most useful parameter for detecting AKI and monitoring progression of CKD.\textsuperscript{138,139} For this reason, and others mentioned previously, there is a need for a sensitive biomarker of KD to detect kidney injury prior to increases in SCC and irreversible changes.

Several serum or urinary biomarkers have been evaluated in cats but none have yet shown advantage beyond SCC. N-acetyl-β-D-glucosaminidase (NAG), a protein secreted by renal proximal tubules, was evaluated in healthy cats, cats with CKD, cats with lower urinary tract disease, and one cat given a nephrotoxic dose of sulfonamide.\textsuperscript{140}
There was no significant correlation between SCC and urinary NAG or SUN. Urinary NAG increased in the cat administered the nephrotoxin, as did SCC. Likewise, Jepson et al.\textsuperscript{18} investigated the utility of NAG measurement in cats with and without azotemia.

NAG was detected in urine but levels were not predictive of azotemia. NAG measurement was also investigated as a biomarker of CKD in cats with hyperthyroidism. Baseline NAG did not differentiate cats with from those without ‘masked azotemia’ prior to treatment of hyperthyroidism, although urinary NAG was increased in cats that became azotemic post treatment.\textsuperscript{141} Cauxin, a protein produced by proximal tubules and measured in urine, was investigated in azotemic cats, but a relationship with SCC or UPC ratio was not found.\textsuperscript{142}

Retinol binding protein (RBP) is a potential marker of tubular dysfunction that is produced by hepatocytes, filtered across the glomerulus, and re-absorbed by proximal tubules. RBP measurement in urine has been validated in cats but was not predictive of presence of azotemia after treatment of hyperthyroidism.\textsuperscript{125,143} However, RBP concentration decreased in hyperthyroid cats that were not azotemic after therapy, suggesting there may be renal tubular dysfunction during hyperthyroidism. Plasma asymmetric dimethylarginine (ADMA) concentration was increased in cats with CKD and correlated with SCC but was not superior to measurement of SCC.\textsuperscript{144} It is possible some biomarkers have not been adequately investigated in cats or that they may be useful if measured at different times or under different conditions. For example, it may be more appropriate to measure NAG early in AKI before increase in SCC rather than in stable CKD or hyperthyroidism.
Biomarkers of Human Kidney Disease: Kidney Injury Molecule 1

Concurrent with development of the AKI scoring system, the American Society of Nephrology designated the identification, characterization and development of new biomarkers of renal disease as a key area of research, and called for a collaborative effort to organize research around the Acute Kidney Injury-Clinical Initiative.\(^\text{145}\) The search for biomarkers was based on identifying different pathophysiological causes, regions of kidney insult, and AKI versus CKD. Desirable biomarker characteristics should be high sensitivity and specificity across a wide range of etiologies, change prior to increased SCC, correlation with severity of injury, prognostic utility and ease of measurement.\(^\text{146}\)

Of approximately 40 urinary and serum biomarkers of KD being evaluated in 2007 for potential clinical use to detect nephrotoxicity, eight urinary biomarkers were selected for testing in preclinical rodent nephrotoxicity studies and human clinical observation trials by the US Food and Drug Administration (FDA), the European Medicines Agency and Pharmaceuticals (EMAP) and the Medical Devices Agency (Japan).\(^\text{51,147,148}\) One of those biomarkers was kidney injury molecule-1 (designated KIM-1 in humans and Kim-1 in rodents).\(^\text{149}\) First reported in 1998, KIM-1 is a type I transmembrane glycoprotein expressed in proximal renal tubular epithelial cells after ischemic injury to kidneys, and shed into urine.\(^\text{150,151}\) The protein was identified in kidney tissue by representational difference analysis of mRNA populations in regenerating and normal kidneys of rats with unilateral renal ischemia and reperfusion.\(^\text{150}\) Kim-1 was dramatically up-regulated in the ischemic kidney compared to the sham operated kidney. Immunohistochemistry (IHC) demonstrated the protein was expressed in proliferating
bromodeoxyuridine-positive cells and co-localized with vimentin in dedifferentiated cells. Kim-1 was abundant within the apical membrane of the S3 segment of proximal renal tubule cells in the ischemic kidney but sparse in the unaffected kidney. DNA sequencing found KIM-1 was 85% similar to monkey HAVCR1 (hepatitis A virus cellular receptor-1). Subsequently, in humans two splice variants of KIM-1, KIM-1a and 1b, with identical extracellular domains but different intracytoplasmic domains and tissue distributions, were identified. The transcript KIM-1b (known as KIM-1) was expressed predominantly in human kidney cells, and the extracellular domain of the protein was cleaved by a metalloproteinase and released into supernatant of cultured cells. KIM-1 is a type 1 transmembrane glycoprotein of 359 aa. The extracellular portion consists of 270 aa with a signal sequence, a six-cysteine immunoglobulin (Ig)-like domain, three potential N-glycosylation sites and a tyrosine, serine, proline-rich domain characteristic of a mucin-like domain. There is a 21 aa transmembrane domain and a 48 aa intracytoplasmic domain with a conserved tyrosine phosphorylation signalling motif. Structurally, KIM-1 most closely resembles mucosal addressin cell adhesion molecule 1. Rat Kim-1 consists of 307 aa with a shorter mucin domain, but retention of the same other domains and features as human KIM-1. Two Kim-1 isoforms with similar structural domains have been shown in mice, and three isoforms in canine cultured kidney cells.

KIM-1 was extensively expressed on the apical aspect of proximal tubule epithelial cells in biopsy samples from patients with ATN due to ischemia, but was not detected in normal kidney tissue of patients with nephrectomy associated with renal carcinoma.
the same study, KIM-1 was abundant in urine of patients with AKI due to ATN, and to a lesser extent in urine of patients with KD due to other forms of injury. Other urinary substances measured concurrently such as γ-glutamyltransferase, alkaline phosphatase and protein did not discriminate ATN from other forms of renal injury. It was suggested KIM-1 might serve as a useful biomarker for early diagnosis of renal injury since urinary KIM-1 (uKIM-1) was found in one patient within 12 hours of the ischemic insult.156

Mice with PKD markedly up-regulated Kim-1 compared to control mice.149 Kim-1 was present in a subset of cysts and in clusters of proximal tubules near cysts, and cells in the interstitium surrounding Kim-1-expressing tubules had high proliferative activity. It was therefore postulated that Kim-1 expression in tubules is strongly associated with dedifferentiation of epithelial cells and may contribute to development of interstitial fibrosis.149

In 2004, it was suggested Kim-1 in urine might serve as a general biomarker for renal tubular injury.157 Hence, Kim-1 was evaluated as a biomarker for renal injury from three types of nephrotoxicants in the rat: S- (1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC), folic acid and cisplatin. Marked increases in tissue Kim-1 expression were confirmed by immunoblotting in all three models. The protein localized to proximal tubule epithelial cells, and was detected in urine of toxicant-treated rats prior to increases in SCC.157

In 2005 a quantitative test was developed to detect the Kim-1 ectodomain in rodent urine, and the test was applied in models of ischemia/reperfusion kidney injury and cisplatin nephrotoxicity.158 Increases in uKim-1 concentration occurred earlier than
changes in biomarkers such as SCC, SUN and urinary NAG, glucose and protein. Urinary Kim-1 also increased with incremental cisplatin dose increases and duration of ischemia. The authors concluded that Kim-1 measurement was reproducible in more than one model of kidney injury and more sensitive than other markers of renal function. They suggested uKIM-1 measurement would be useful in pathophysiological studies and preclinical nephrotoxicity assessment.$^{158}$

The relationship between urinary NAG and uKIM-1 concentration and adverse clinical outcomes was prospectively evaluated in a cohort of 201 hospitalized patients with acute renal failure. Concentration of both molecules predicted adverse clinical outcomes in patients with ARF using the Acute Physiology and Chronic Health Evaluation (APACHE) II and Multiple Organ Failure score.$^{159}$ In a study of 102 patients with different renal diseases, concurrent uKIM-1 was measured and renal biopsies were obtained.$^{160}$ Sequential dual immunohistochemical staining for KIM-1, macrophages, α-smooth muscle actin, aquaporin-1 (AQP 1, proximal tubular marker), E-cadherin (distal tubular marker) and vimentin (dedifferentiation marker) showed increased KIM-1 expression in proximal tubular cells in all types of renal disease and in association with inflammation, fibrosis and dedifferentiation. uKIM-1 concentration was significantly increased in all diseases and correlated positively with the degree of kidney injury and negatively with creatinine clearance and estimated GFR, and was not associated with proteinuria. KIM-1 was not expressed in atrophied tubules. The authors concluded uKIM-1 reflects tissue KIM-1 concentration, and based on these results it was suggested uKIM-1 could be a non-invasive biomarker of renal disease in humans.$^{160}$ Since then,
various studies have indicated KIM-1 may be a useful urinary biomarker to allow for the diagnosis of early naturally occurring AKI before increases in SCC. In a study of children undergoing cardiac surgery, uKIM-1 and urine NAG concentration increased before SCC in patients who experienced AKI.\textsuperscript{161} Urine KIM-1 was found to be a sensitive marker for allograft injury or rejection in renal transplant patients, and correlated well with histological changes in protocol-driven biopsies.\textsuperscript{162} However, in the same study KIM-1 immunoreactivity was also noted in 28\% of biopsies without detectable histological evidence of tubular injury. The implementation of cut-off values for IHC was suggested by one critic, as overlap in KIM-1 immunoreactivity among histopathologically normal and abnormal kidney tissue could be problematic and might result in unnecessary additional biopsies if used as the sole marker.\textsuperscript{163} However, in a rebuttal, Bonventre concluded that KIM-1 immunoreactivity was more sensitive than routine histology for detection of early kidney injury, as KIM-1 expression positively correlated with SCC and SUN and inversely correlated with GFR.\textsuperscript{164} These conclusions summarized what may be a major strength of detecting KIM-1 in urine since increases prior to histological changes might allow intervention before renal tubular cells are irreversibly injured, which in turn might halt progression to CKD.

In a recent prospective study of 63 patients with suspected allograft rejection or worsening renal disease over 39.7 months, the rate of renal functional decline significantly correlated with uKIM-1 concentration. At 48 months, the graft survival rate for high and low KIM-1 groups was 46.2 and 78.6\%, respectively. After adjusting for confounding variables, each log of higher uKIM-1 concentration conferred a 2.9-fold
higher risk of developing graft failure, further supporting uKIM-1 concentration as a prognostic indicator of renal function. \(^{165}\) In a prospective study of 90 patients undergoing cardiovascular surgery, AKI was detected in 36 patients as defined by an increase in SCC at 72 hours post-surgery. Urinary NGAL, NAG and KIM-1 all increased within 24 hours prior to changes in SCC. \(^{166}\) The combined panel of markers had better predictability than any one marker, possibly because each marker localizes to a different area of the kidney. A rapid urine KIM-1 lateral-flow assay for both humans and rats, the Rena-strip™ (Bioassay Works, Ijamsville, MD), was recently validated in human and rat preclinical trials, generating results within 20 minutes. In addition, strips can be read to quantify the amount of KIM-1 using a chromatographic scanner. \(^{167}\) Human KIM-1 was stable in urine at various pH’s and after freezing at -80°C. \(^{168,169}\)

In 2010, KIM-1 became the first biomarker qualified by the FDA and European Medicines Agency and Pharmaceuticals European Medicines Agency and Pharmaceuticals for kidney toxicity studies. \(^{170}\) In a multi-center study, Kim-1 expression in rats was very low to absent in all organs at baseline. Only kidney had greater than 100-fold Kim-1 induction after toxic injury, demonstrating organ specificity. Kim-1 outperformed SCC, SUN and urinary NAG concentration in terms of early detection, specificity and sensitivity compared to histopathological changes after ischemic and toxic renal tubular injury. \(^{170}\) At present there are at least 20 active, recruiting or just completed clinical trials in people evaluating KIM-1 in critical disease states and prerenal azotemia (ClinicalTrialsFeeds.org, accessed March 2014).

Functions of KIM-1 are incompletely understood and controversial. KIM-1 likely
has specific functions rather than being just a marker of tubular injury. The extracellular immunoglobulin (Ig)-like domain likely mediates adhesion of tubular epithelium to matrix\textsuperscript{171} and forms a highly specific recognition site for ligands such as phosphatidylserine, which is expressed on apoptotic cells.\textsuperscript{152} The mucin-like domain in KIM-1 is thought responsible for cell-to-cell adhesion.\textsuperscript{151} A highly conserved cytoplasmic tyrosine kinase phosphorylation motif likely functions in signal transduction and possibly immune responses.\textsuperscript{152,172,173} KIM-1 is co-expressed with de-differentiation and proliferation markers in regenerating proximal tubule cells after ischemic injury in rats and humans, and is believed to contribute to the regenerative response.\textsuperscript{150,151,174} But persistent expression of KIM-1 may contribute to failure of differentiation, and therefore to persistent inflammation and fibroblast proliferation leading to CKD.\textsuperscript{79} In addition, KIM-1 expressing tubular cells that have survived ischemic damage, and cultured canine renal epithelial cell lines induced to express KIM-1, developed a phagocytic phenotype. Cells subsequently recognized phosphatidylserine on apoptotic cells, and internalized apoptotic bodies of tubular debris, which is crucial for renal remodeling and cell restoration.\textsuperscript{175} It has been hypothesized that shedding of the KIM-1 ectodomain into urine may contribute to ligand binding during repair of tubules, or may form a protective layer in the proximal tubular lumen to shield cells from protein casts.\textsuperscript{176} Cleavage of KIM-1 at the juxtamembranous region and release into urine is mediated by matrix metalloproteinase-3 (MMP-3)\textsuperscript{177}, which is activated by reactive oxygen species generated during ischemia and/or inflammation.\textsuperscript{177,178}

Expression of KIM-1 has yet to be fully investigated in humans with co-morbidity.
inflammatory diseases, diabetes, hypertension or CKD, but findings to date suggest release into urine is not increased unless there is concurrent kidney damage or microalbuminuria.\textsuperscript{179,180} KIM-1 is very similar to T-cell immunoglobulin and mucin domain-containing protein-1 (TIM-1), a molecule expressed in a subpopulation of T-lymphocytes. TIM-1 has been proposed to be a co-stimulatory molecule for immune cells, although the potential role has not been elucidated and was considered controversial.\textsuperscript{152,181,182} Co-localization of KIM-1 with vimentin, smooth muscle actin and osteopontin has lead some authors to propose KIM-1 induction as an intermediary step in the development of tubulointerstitial damage and inflammation.\textsuperscript{149,160,183}

\textbf{Summary}

Measuring uKIM-1 is a valuable indicator of tubular damage in naturally occurring kidney disease in humans and in a range of experimental kidney diseases of rodents. In both acute and chronic renal toxicity, and in acute ischemia reperfusion, the S3 segment of the rat proximal tubule was most susceptible to injury.\textsuperscript{61,184-186} This site corresponds to most intense expression of KIM-1 in humans and rats.\textsuperscript{150} AQP-1, a molecule essential for water movement across membranes, co-localizes with KIM-1 in proximal tubules in humans.\textsuperscript{160} In an ischemia reperfusion model of AKI in cats, microscopically identifiable ATN characterized by proximal tubular epithelial karyorrhexis and karyolysis, and formation of tubular casts, was noted.\textsuperscript{114} Cell regeneration was indicated by anisokaryosis, increased cellular basophilia and frequent mitoses. Proximal tubular changes were consistently more severe than glomerular changes. This suggests that proximal renal tubules in cats are a focus of insult in AKI, which could then lead to CKD.
It is possible that small repeated occurrence of AKI without clinical manifestation, or ‘prerenal azotemia’, result in cumulative loss of functional renal mass over the lifetime of a cat, and eventually manifest as CKD. For this reason it is imperative to detect kidney injury early so further injury can be prevented. The utility of measuring KIM-1 as an indicator of kidney injury has not yet been evaluated in cats. However, considering the similarity of KIM-1 sequences from mice, rats, monkeys and humans, there is reason to expect KIM-1 will be readily identifiable in cats. Furthermore, localization of ischemic damage to similar regions in the cat kidney as in human and rat kidneys suggests KIM-1 may be suitable molecule for detecting tubular injury in cats. Hence, it is hoped that identification of KIM-1 in the urine of cats with AKI may lead to earlier or more specific interventions to help prevent further exacerbation of KD. Cats who have an episode of AKI could be identified as ‘at risk’ during subsequent diagnostic, therapeutic or anesthetic procedures. Such cats could be monitored for factors such as hypertension, proteinuria and inflammatory disease that may pose a risk for progression to CKD. In this thesis the structure and expression of KIM-1 in healthy and diseased cats was investigated.
HYPOTHESES

The following hypotheses were addressed: 1) KIM-1 of cats is similar to that of other species; 2) KIM-1 is expressed in specific segments of proximal renal tubule cells of cats with naturally occurring and experimentally induced kidney disease; and 3) KIM-1 is present in urine of cats with acute and chronic kidney injury.

The specific objectives were to 1) characterize genomic and mRNA KIM-1 sequences by designing primers based on conserved regions in other species; 2) immunohistochemically characterize expression of KIM-1 in the nephron of healthy cats and cats with kidney injury; and 3) assess currently available commercial assays for detection of urinary KIM-1.
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Chapter Two

Characterization of kidney injury molecule-1 in cats

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**Short title:** KIM-1 in cats

**Keywords:** Feline, serum creatinine, renal disease, urinalysis

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Abstract

**Background:** Kidney disease (KD) is common in older cats and presumed to arise from subclinical kidney injuries throughout life. Sensitive markers for detecting kidney injury in cats are lacking. Kidney injury molecule 1 (KIM-1) is a sensitive and specific biomarker of kidney injury in humans and rodents.

**Hypothesis/Objectives:** The hypothesis that feline KIM-1 is similar to that of other species, expressed in kidney, and shed into urine of cats at risk of acute kidney injury, was addressed. The objectives were to characterize feline KIM-1 gene and protein, to assess available immunoassays for detection of KIM-1 in urine of cats with a range of disorders, and to identify KIM-1 expression in kidney sections by immunohistochemistry.

**Animals:** Samples from 36 hospitalized and 7 clinically healthy cats were evaluated. Hospitalized cats were divided into 2 groups based on absence (n=20) or presence (n=16) of historical KD.

**Methods:** Feline KIM-1 genomic and complementary DNA sequences were amplified, sequenced and analyzed to determine the presence of isoforms, exon-intron organization and similarity with KIM-1 orthologous sequences. Presence in urine was evaluated by immunoassay and expression in kidney by immunohistochemistry.

**Results:** Expression of 3 feline KIM-1 transcript variants comprising 894, 810 and 705bp were identified in renal tissue. KIM-1 immunoassays yielded positive results in urine of cats with conditions associated with acute kidney injury but not in cats with chronic KD.
Immunohistochemical staining of kidney section revealed KIM-1 staining in proximal tubular cells of cats with positive urine immunoassay results.

**Conclusions and clinical importance:** KIM-1 was expressed in specific segments of the feline nephron and positive immunoassay results were obtained with urine of cats at risk of acute kidney injury. Detection of urine KIM-1 in cats may be a useful indicator of proximal tubular injury that warrants further investigation.
Introduction

Chronic kidney disease (KD) is a common condition in older cats of unknown etiology. Chronic KD has been defined as structural or functional impairment of 1 or both kidneys for longer than 3 months.\(^1\) Surveys have shown incidence of 15.3% in cats older than 15 years of age, and more recently 27.2% in cats older than 10 years.\(^2,3\) In another study, during 1 year of observation, KD developed in 30.5% of clinically healthy cats over 9 years of age.\(^4\) Thus, KD is very common in older cats. While some causes of KD such as urethral obstruction and ethylene glycol or lily toxicity are well known, in many cases the cause of KD is unknown and may be multifactorial.\(^5\) Progression of chronic KD in cats may be unapparent or very gradual.

In human medicine, acute kidney injury (AKI) has replaced the term acute renal failure. AKI has been proposed to encompass the entire spectrum from minor change in renal function to requirement for renal replacement therapy.\(^6\) Thus, the change in terminology is meant to imply that acute injury may progress to chronic KD or may result in recovery. In humans, AKI most often occurs concurrent with other disease, and risk factors for AKI are ill defined but even transient azotemia is associated with increased mortality.\(^7,8\) AKI Network (AKIN) criteria define AKI as a reduction in kidney function over 48 hours associated with an absolute increase of $\geq 26.4 \ \mu \text{mol/L}$ ($\geq 0.3 \ \text{mg/dL}$) or a percent increase of $\geq 50\%$ (1.5-fold from baseline) in serum creatinine (SC) concentration, or a reduction in urine output.\(^6\) Thus, by these criteria relative increases in SC may be small and remain within SC reference interval (termed “critical difference”) but nevertheless indicate AKI.
AKI is infrequently identified in cats, which may be due to limitations of current diagnostic approaches or due to the subclinical nature of many kidney injuries in cats. Reviews of AKI in cats have addressed causes of clinical signs of KD but understanding of the causes of subclinical injury remains incomplete. It is plausible that in cats, as in people, episodic subclinical injury or clinical AKI with apparent recovery progress to chronic KD. However, ability to diagnose AKI and to prognosticate KD in cats is very limited. SC concentration is the most widely used biomarker for KD, but is an insensitive and imperfectly specific indicator since azotemia develops only after approximately 75% reduction in renal function. A range of other potential biomarkers has been assessed in cats, but to date none has predicted progression of renal disease better than SC concentration. In cats and people, SC concentration varies between individuals, and critical differences within the same individual rather than relative to a population without KD may be more meaningful. Recently, approaches for the diagnosis of AKI in cats and dogs similar to AKIN criteria in people have been proposed. These approaches incorporate a range of clinical and laboratory variables, but limitations due to lack of sensitive indicators of AKI remain.

Kidney injury molecule 1 (KIM-1), also known as hepatitis A virus cell receptor 1 (HAVCR1) and T cell immunoglobulin 1 (TIM-1), is a renal tubular transmembrane glycoprotein thought to function in cell-to-cell or cell-to-matrix adhesion. KIM-1 has characteristics that make it a useful biomarker for AKI in people and rodents: low expression in healthy kidneys, rapid 3- to 100-fold increase after ischemic or toxic injury and release of an extracellular portion into urine. Urine KIM-1 concentration
correlated with severity of AKI and decreased as kidney repair progressed. For these reasons, KIM-1 may also be a useful marker of AKI in cats. Hence, the goals of this study were to identify and characterize feline KIM-1 and to investigate expression and measurement in health and disease.

**Materials and Methods**

**Sequence Data Sources**

The following KIM-1 nucleotide and amino acid sequences were obtained from the National Center for Biotechnology Information (NCBI) database: *Homo sapiens*, NP_036338.2; *Canis lupus*, NP_001192043.1; *Mus musculus*, NP_599009.2; *Rattus norvegicus*, NP_775172.1; and *Pan troglodytes*, XP_001135569.1 (all accessed January 2014). *Felis catus* genomic sequence was obtained from the Genome Annotation Resource Fields (GARFIELD) feline database. Multiple sequence alignments were performed with Geneious Pro software (V5.5.3, Biomatters, Auckland, New Zealand) to identify areas of similarity, which were then further investigated using the Basic Local Alignment Search Tool (BLAST) and Constraint-Based Multiple Alignment Tool (COBALT) provided by NCBI. Conserved regions of KIM-1 were selected for primer design.

**Amplification of Feline KIM-1 cDNA**

Fresh feline kidney tissue was obtained from a cat euthanized for causes unrelated to this study, immersed in RNAlater (Qiagen, Mississauga, ON) and frozen at -80°C.
RNA purification was performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. Complementary DNA (cDNA) was synthesized with Superscript III Reverse Transcriptase (Invitrogen, Mississauga, ON). KIM-1 cDNA variants including complete open reading frames (ORF) were amplified by PCR with KIM-1 forward (5’-GGC ACA CCT ACC AGT CTG CTT-3’) and KIM-1 reverse (5’-CTG TCT TCT GCA GTC AAG GG-3’) primers (Sigma Aldrich, Burlington, ON). PCR amplifications were carried out with HotStarTaq Plus DNA polymerase (Qiagen) in a final volume of 20 µL, including 10 µL of 2x HotStarTaq Plus Master Mix PCR buffer, 0.2 mM MgSO₄, 0.5 µM of each primer, and 2 µL of template DNA. Conditions for amplification were 1 min at 94°C followed by 40 cycles of 94°C for 30s; 57°C for 30s; and 72°C for 90s with a final extension at 72°C for 7 min. PCR products were separated by electrophoresis and bands of appropriate size were excised from the gel, purified using the QIAquick Gel Extraction Kit (Qiagen), and submitted for automated sequencing (Laboratory Services Division, Guelph, ON).

**Characterization of the Feline KIM-1 Gene**

The feline cDNA sequence was aligned with the canine KIM-1 genomic sequence (GenBank AAEX03003064.1) to predict exon-intron boundaries. Multiple potential exons in the cDNA were identified, and a strategy was designed to sequentially amplify all introns and exons with specific primers (Table 1) and to generate a contiguous consensus sequence. Optimized conditions for amplification were 5 min at 94°C followed by 40 cycles of 94°C for 30s; 58°C for 30s; and 72°C for 3 min with a final extension at 72°C for 7 min. PCR products were isolated by electrophoresis, purified
and sequenced. Based on analysis of sequences obtained, additional primers were designed as needed to complete amplification of the entire gene. Individual overlapping sequences were assembled with Geneious Pro software.

**Urine Samples and KIM-1 Immunoassay**

Aliquots of urine remaining after urinalysis was performed in the Animal Health Laboratory (AHL, University of Guelph) were collected. Samples from cats in the Intensive Care Unit (ICU) of the Ontario Veterinary College Health Sciences Centre with concurrent complete blood cell count (CBC) and serum biochemical results were selected. Samples were voided into non-absorbent litter or collected via catheter or cystocentesis, as indicated by attending clinicians. Where possible, subsequent urine samples were also collected. Urinalyses were performed using an automated urine dipstick reader and semi-quantitative microscopic sediment analysis. All serum assays were performed on a Cobas 4800 biochemistry analyzer (Roche, Mississauga, ON). Urine cultures were requested at the discretion of clinicians. Cases were categorized as critically ill with no history of KD (group A) or critically ill with history of KD (group B). Assignment of prior KD was based on historical laboratory results indicating SC concentration above reference interval (RI) and urine specific gravity (USG) <1.035 over a period of more than 1 month. Samples from cats with equivocal history or incomplete blood profiles were excluded. Urine and blood samples collected from non-hospitalized cats without illnesses were analyzed as above (approved by the Institutional Animal Care Committee, protocol 10R030). These cats (group C) had CBC and biochemical results within laboratory RI and USG >1.035. All urine samples were
stored in plastic tubes without additives (BD Vacutainer tubes, BD Diagnostics, Franklin Lakes, NJ) at 4°C for up to 24 hours before freezing at -80°C. KIM-1 in urine was detected with a lateral flow device designed to detect rat KIM-1 (R-Rena-Strip, BioAssay Works, Ijamsville, MD) as per the manufacturer’s instructions with color reactions read at 20 minutes. This point-of-care assay utilizes gold nanoparticles impregnated with KIM-1 antibody on a multilayer membrane with capillary action. Results were classified as “positive” or “negative” according to presence or absence of a color reaction in the test sample and if the positive control reaction yielded a color reaction.

**KIM-1 Immunohistochemistry**

Expression of KIM-1 in kidney tissues was investigated by immunohistochemistry (IHC). Five-μm thick paraffin-embedded sections were deparaffinized in xylene, rehydrated in graded alcohols, incubated consecutively for 10 min with dual endogenous enzyme blocker, 30 min with serum free protein blocker, overnight with an optimized 1/40 dilution of antibody to KIM-1 human MAb Clone 219211 (R&D Systems, Minneapolis, MN) and 30 min with Envision Dual Link System-HRP (DakoCytomation, Mississauga, ON). Bound antibodies were detected with Nova Red chromogen (Vector Laboratories, Burlington, ON), and slides were counterstained with hematoxylin. Each batch of slides included negative (omission of primary antibody or pre-incubation of antibody with human KIM-1 peptide for 2 hours at dilution of 1:40 [R&D Systems]) and positive controls (sections from a cat with
acute tubular necrosis). KIM-1 immunohistochemical staining was assessed as positive or negative.

Results

Characterization of the Feline KIM-1 cDNA and Gene

The feline genome database GARFIELD was searched using the key words KIM-1 and HAVCR1.\textsuperscript{28,29} Chromosome A1 contained 4 coding regions with similarity to HAVCR1 that were imported into Geneious Pro software. Regions were aligned with HAVCR1/KIM-1 sequences of rat, dog, mouse and human using BLAST and COBALT. A region highly similar to KIM-1 of other species was identified in 1 of the feline sequences. Sequential application of primers that encompassed start and stop codons to kidney cDNA yielded 3 amplicons termed isoform A, B and C, which corresponded to 894, 810 and 705bp, respectively (Fig 1). Three polymorphic nucleotides differentiated isoform C from A and B. Overall identity was 90.6% for isoforms A and B, and 78.5% for isoforms A and C. Sequences were deposited in NCBI GenBank with accession numbers KF540032, KF540033 and KF540034.

Eight exons were predicted by alignment of feline cDNA sequences with canine KIM-1 genomic DNA. To amplify the entire feline KIM-1 gene, a first set of primers was based on a related canine genomic sequence, and subsequent primers (Table 1) were designed sequentially to match newly derived sequences until the entire gene sequence of 21,059bp was determined. Alignment of the feline cDNA sequence with the full
feline genomic DNA sequence indicated 8 exons in isoform A, 7 in isoform B and 6 in isoform C (Fig 2).

**Functional Motifs of Feline KIM-1**

*In silico* translation of the full-length feline cDNA yielded a protein of 297 amino acids (AA). Analysis of this sequence for conserved domains using tools of the NCBI Conserved Domains Database indicated presence of an immunoglobulin (Ig)-like domain with 6 cysteines, a mucin-like domain rich in threonines, serines and prolines, a transmembrane domain, and a short cytoplasmic domain containing a conserved tyrosine motif (Fig 3). Similar motifs are present in the rat and human KIM-1 protein. Presence of 14 asparagines with specific adjacent AA in the cytoplasmic domain indicated 4 possible glycosylation recognition sites. Absence of exon 3 in isoform B, and exons 3 and 4 in isoform C, results in progressive loss of the mucin-like domain such that isoform C contains few threonines, serines and prolines. In isoform C, alanine at position 37 is replaced by threonine, phenylalanine at position 237 by leucine, and serine at position 245 by isoleucine. Thus, isoform C differs at 3 positions from isoform A and B.

**Phylogenetic Analysis**

Amino acid alignment (Geneious Pro, blocks substitution matrix [BLOSUM] = 65, gap open penalty = 12, gap extension penalty = 3, free end gap setting) of KIM-1 isoform A with the corresponding dog, human, mouse and rat sequences (GenBank
ADZ24777, NP_036338, NM_599009 and NM_775172, respectively, accessed January 28, 2014) indicated 83.2%, 43.8%, 44.7% and 43.7% identity, respectively. Calculation of phylogenetic relationships (Geneious Pro, Jukes Cantor model, neighbor-joining method) with the mallard (Anas platyrhynchos) KIM-1 sequence (GenBank XP_005029962) as an outlier confirmed closest relationship of cat KIM-1 with dog KIM-1 and relatively greater distance to horse, human and rodent sequences (Fig 4).

**Urine Samples**

Urine samples were available from 36 ill and 7 clinically healthy cats. Of the ill cats, 20 had no evidence of KD prior to admission (group A) and ranged in age from 1 to 17 years (mean 7.3 years, Table 2). These cats had a variety of illnesses with the potential to cause AKI. First biochemical evaluation in ICU indicated SC concentration above RI in 4 cats (RI 50-190 µmol/L, 0.57-2.15 mg/dL). In 15 cats USG was <1.035, which was likely due to fluid administration since urine samples were inconsistently collected prior to initiation of therapy.

Sixteen ill cats had historical KD (group B) as indicated by persistently increased SC concentration and USG <1.035, and ranged in age from 0.5 to 17.0 years (mean 9.7 years). First biochemical evaluation during hospitalization revealed SC concentrations >100 µmol/L above historical values in 8 cats, which was ascribed to recent AKI superimposed on chronic KD. Five cats had SC concentration within RI upon admission but had received fluid therapy prior to referral, or had developed hyperthyroidism in addition to KD. In this group of 16 cats, SC concentration ranged from 85 to 1238 µmol/L (0.96-14.00 mg/dL) (Table 2).
Seven clinically healthy cats had no history of KD and ranged in age from 1 to 3 years. Three were neutered males, 3 were intact males and 1 was an intact female. SC concentration ranged from 49 to 125 µmol/L (0.55-1.41 mg/dL). USG on admission ranged from 1.039 to 1.064, urine protein from 1 to 3 (semiquantitative on dipstick), all samples were negative for urine glucose, and hemoglobin reactions ranged from 0 and 2.

**Urine KIM-1 Immunoassay**

The urine immunoassay (UIA) test completion entailed approximately 20 minutes, and test positive controls consistently yielded the expected reaction (Fig 5). Eight positive KIM-1 UIA results were obtained from 7 cats of group A (critically ill, no prior KD). Two cats had SC concentration that increased during hospitalization but remained within RI, conditions potentially associated with hypoperfusion (severe pancreatitis and advanced myocardial disease, respectively) and positive KIM-1 UIA results following initial negative UIA results. Four cats had SC concentration within RI but positive UIA results on admission, and 2 of these were re-tested within a few days and negative UIA results were obtained. One cat had increased SC concentration due to urethral obstruction lasting several days and 2 consecutive positive UIA results. After a few days a repeat UIA result was negative and SC concentration was within RI.

Thirteen cats of group A had negative KIM-1 UIA results on admission. Four of these 13 cats were euthanized in hospital due to untreated underlying conditions and 1 died at home 6 days post discharge. Two of the 13 cats had ingested *Lilium* 4 or more days prior to referral.
Results of KIM-1 UIA were positive on admission in 4 of 16 cats of group B (critically ill, prior KD). Two of these 4 cats had terminal cancer, 1 had hyperthyroidism and 1 had unresponsive acute exacerbation of chronic KD. Two cats had a subsequent negative UIA result concurrent with decreased SC concentration. Twelve cats of group B had single or multiple negative UIA results.

Samples from all cats of group C yielded negative UIA results.

**KIM-1 Immunohistochemistry**

Kidney sections were evaluated from all cats submitted for post mortem examination or from biopsies obtained ante mortem. Thus, wedge kidney sections including cortex and medulla were available from 4 cats of group A and five cats of group B. From 2 additional cats of group B, Tru-cut biopsies comprised only of cortex were available. Sections from group A cats had variably severe acute tubular necrosis but no evidence of chronic KD (fibrosis, glomerular sclerosis and/or inflammatory cells).\(^{31}\) KIM-1 IHC staining was consistently detected in proximal convoluted tubules from cats with acute tubular necrosis, and abrogated by pre-incubation of the antibody with KIM-1 peptide or omission of antibody (Fig 6A). KIM-1 staining was considered to be specific due to lack of staining in sections where antibody was pre-incubated with KIM-1 peptide, and absence of KIM-1 staining of glomeruli, endothelium and medullary regions (Fig 6A). Staining in sections from cats of group A was typically prominent in specific tubules of the outer stripe of the outer medulla (OSOM), and also noticeable in luminal cell debris (Fig 6B). The OSOM contains the distal segment of the
straight portion of the proximal tubule, the collecting ducts and descending portion of
the loop of Henle located near the cortico-medullary junction.\textsuperscript{32}

Sections from cats of group B had evidence of chronic KD such as multifocal fibrosis, interstitial nephritis, glomerulosclerosis and/or tubular atrophy. KIM-1 staining in sections from 3 cats with positive KIM-1 UIA results was variably intense and confined to occasional tubules of the OSOM and luminal cell debris (Fig 6C). Sections from one cat with multiple negative UIA results, chronic KD, severe fibrosis and interstitial inflammation had no tubular staining for KIM-1 (Fig 6D). Biopsy sections also showed no KIM-1 immunostaining, however, these sections lacked the OSOM.

At high magnification, tubules from cats with AKI due to potential hypoperfusion and ischemia showed variable staining, with absence of KIM-1 staining in intact cells with brush borders suggestive of proximal tubular cells, and moderately intense staining in cells with pyknotic nuclei (Fig 7).

Discussion

Urine KIM-1 is a promising biomarker of kidney injury and KD in humans. KD is very common in cats but sensitive, specific and non-invasive biomarkers of KD or injury are lacking. Hence, goals of this study were to characterize the feline KIM-1 gene and protein, and to assess the utility of available KIM-1 assays in cats at risk of kidney injury and with naturally occurring KD.

Feline KIM-1 cDNA amplified from kidney tissue of cats had similar structure to that of human, rat, mouse and dog, and contained a cytoplasmic motif highly conserved
across species. Analogous to dogs, cats have 3 isoforms of feline KIM-1 cDNA while mice have two isoforms, and humans and rats have only one.\textsuperscript{23,24,33,34} Functions of the different isoforms have not been determined, but exon-intron structure of cDNA predicted from genomic KIM-1 sequences suggests derivation from alternative splicing. Exon deletion appears to be the most common mechanism that gives rise to such isoforms.\textsuperscript{35} Since KIM-1 may also be expressed by lymphocytes, which can be present in kidney tissue, it is conceivable that there may be cell- and tissue-specific expression and unique functions of different isoforms.\textsuperscript{36} Furthermore, KIM-1 isoforms could be expressed differentially in specific regions of the nephron or in response to different types of injury. Non-synonymous SNPs in isoform C result in 3 AA changes, and 2 of these AA have different biochemical properties, suggesting that this isoform may have subtle functional differences. SNPs have not been investigated regarding population frequency or breed association.

Conservation of certain KIM-1 motifs across multiple species suggests similar functions. The extracellular Ig-like domain likely mediates protein-protein interactions at the cell surface, and cell-extracellular matrix adhesion.\textsuperscript{37} The cysteine sites allow folding of the Ig-like domain to form recognition sites for ligands such as phosphatidylserine expressed on apoptotic cells.\textsuperscript{38} The mucin-like domain in full length KIM-1 is likely responsible for cell-to-cell adhesion.\textsuperscript{24} Absence of a section of the mucin-like domain in isoform C suggests this isoform may have lost cell-cell adhesive function. A highly conserved cytoplasmic tyrosine kinase phosphorylation motif with
expected function in signal transduction and immune response was also present in the cat.\textsuperscript{38-40}

Functions of KIM-1 are incompletely understood and controversial. KIM-1 likely has specific functions rather than just being a marker of tubular injury. KIM-1 is co-expressed with de-differentiation and proliferation markers in regenerating proximal tubule cells after ischemic injury in rats and humans, and is believed to contribute to the regenerative response.\textsuperscript{23,24,41} In addition, tubular cells that survive ischemic damage and express KIM-1 develop a phagocytic phenotype, recognize phosphatidylserine on apoptotic cells and internalize such apoptotic cells thereby clearing tubular debris.\textsuperscript{42} Cleavage of KIM-1 at the juxtamembranous region and release into urine is mediated by matrix metalloproteinase-3 (MMP-3).\textsuperscript{43} Ischemia and/or inflammation with generation of reactive oxygen species and activation of MMP-3 induce transcription of KIM-1 and enhance cleavage.\textsuperscript{43,44} Co-localization of KIM-1 with vimentin, smooth muscle actin, and osteopontin has led some authors to propose KIM-1 induction as an intermediary step in the development of tubulointerstitial damage and inflammation.\textsuperscript{41,45,46}

KIM-1 in urine and kidney tissue was evaluated in samples from ill cats divided into two groups according to absence or presence of historical KD. Two cats of group A could have been classified by International Renal Interest Society (IRIS) criteria as having grade I AKI since they had progressive increase in SC concentration of $\geq 26.4 \mu \text{mol/L}$ ($\geq 0.3 \text{ mg/dL}$) but these values remained within RI during a 48 hour interval (www.iris-kidney.com, accessed Feb 2014). Other cats of group A might have experienced AKI during hospitalization, but repeat SC concentrations were not
determined. Fifteen of 16 cats with a history of KD in group B might have been classified as chronic KD stage 2 to 4 by IRIS guidelines (www.iris-kidney.com, accessed Jan 2014). However, since cats were critically ill with unstable glomerular filtration rates (GFR), had variably received fluid therapy, repeat SC concentrations were not available and fasting could not be assured, we chose to indicate the actual SC concentrations rather than stratify a relatively small number of cats into IRIS stages. Several cats had normal SC concentration on admission but had historically increased SC concentration. This change was attributed to increased GFR that developed concurrent with hyperthyroidism or fluid therapy. Thirteen of 16 cats with “critical illness and prior KD” had proteinuria. However, neither blood pressure nor urine protein-to-creatinine (UPC) ratio was consistently determined. Application of IRIS criteria is of benefit for staging KD in stable patients and initiating appropriate therapy. However, IRIS classification is challenging in patients with unstable KD and conditions such as hyperthyroidism. Several cats of group C had proteinuria, which was attributed to hematuria in 2 cases; and presence of sperm, high specific gravity or dry food diets in the other cases. Lack of underlying renal disease could not be entirely ruled out.

KIM-1 positive results were obtained in 11 cats of group A and group B, but in none of the clinically healthy cats. KIM-1 is shed from acutely injured tubular cells in both rats, mice and humans. Therefore, KIM-1 might be expected in urine of cats that experience hypotension, renal hypoperfusion and ischemia, or tubular toxic or inflammatory injury. Although neither the sensitivity of detecting KIM-1 in urine nor the specificity for injury of specific segments of the nephron can be deduced from the
data presented here, KIM-1 was not detected in urine of healthy cats, and was detected in cats who had or may have had kidney injury. Positive KIM-1 UIA results developed in some cats concurrent with rising SC concentration, or became negative concomitant with presumed resolution of hypotension or improvement of critical illness. It has been suggested that severe acute tubular injury may result in rapid and marked cellular KIM-1 up-regulation and then shedding into urine, and that extensive death of tubular cells may result in subsequent lack of detection of KIM-1 in urine or tissues. If the dynamics of KIM-1 are similar in cats, it is conceivable that negative results may indicate large scale loss of tubular cells such as in lily toxicity, or replacement with less differentiated cells during repair. Therefore, it will be important to precisely determine the temporal appearance of KIM-1 in urine in relation to well-defined kidney injury. However, this is difficult to accomplish in studies involving clinical patients.

Kidney tissues for KIM-1 IHC were available from 11 cases. In 8 cases there was positive staining in proximal tubular cells although UIA results had been negative in 3 of these cats. Possible reasons for this discrepancy are that not all KIM-1 expressed by tubular epithelium is also cleaved and shed into urine, that the UIA is less sensitive than IHC, or that the protein is unstable in urine. Ideally, urine samples should be incubated with protease inhibitors and rapidly frozen prior to analysis, which was not feasible in this study for samples collected in a non-invasive manner from critically ill patients. Furthermore, a cat-specific KIM-1 UIA might have been more sensitive and yielded results that agree to a greater extent with IHC results, but is unavailable at this time. Three cats had negative KIM-1 IHC results although they had presumed acute
exacerbation of chronic KD. In 2 of these cats only small biopsies lacking the OSOM were available, therefore the region of the nephron where KIM-1 should be expressed was absent. The third cat had severe chronic fibrotic kidney disease, persistent marked elevation in SC, and several days lapsed between exacerbation of KD and euthanasia. The most likely reason for lack of KIM-1 staining in this case was loss or fibrotic change of proximal tubular cells in an animal with end-stage KD.

Six cats had positive KIM-1 UIA results while their SC concentrations were within RI. This finding is similar to those in rodents and people where injured renal tubules up-regulate and shed KIM-1 into urine prior to significant increases in SC concentration.\textsuperscript{52,57} Furthermore, SC concentration above a population-derived RI is considered to be an insensitive indicator of reduced GFR, and “critical differences” in SC concentration over time within an individual may more accurately reflect changes in GFR.\textsuperscript{16-18,58} Thus, from the limited number of cases included here, it appears that apparent detection of KIM-1 in urine may be a very sensitive indicator of acute injury of tubular cells in cats without pre-existing KD who experience kidney injury due to hypovolemia, hypotension, ischemia, toxic or septic insult, or who have acute injury superimposed on pre-existing KD.

A limitation of this study was that samples from defined AKI were unavailable for use as positive controls. Furthermore, although antibodies in the UIA and IHC were directed to KIM-1 regions similar in cats, rats and humans, sensitivity of both assays would likely be higher with antibodies matching precisely to feline KIM-1 epitopes. Hence, preliminary evaluation of the UIA for rat KIM-1 did not entail proper test
validation, but rather a proof-of-principle to justify future generation of feline-specific reagents. Measurement of KIM-1 gene transcripts by quantitative PCR was not deemed meaningful since induced expression is highly variable across different segments of the proximal convoluted tubule and nephron, and only comparing expression across identical nephron segments would be informative. This study was not meant to evaluate the specificity or sensitivity of non-feline UIA or IHC assay but rather to demonstrate KIM-1 expression in the feline kidney and suggest shedding into urine. Further elucidation of the role of feline KIM-1 in AKI will depend on development of more specific reagents.

In rodents and primates KIM-1 measurement compared favorably in sensitivity and specificity to other urinary biomarkers for detection of AKI. In rat experimental renal ischemia and toxic injury, measurement of urine KIM-1 was more sensitive than SC concentration, BUN, urine glucose or urine N-acetyl-beta-D-glucosaminidase (NAG).59 Similarly, in humans KIM-1 assays outperformed NAG, MMP3 and γ-glutamyl transpeptidase assays for detection of AKI.60 Data regarding performance in companion animals are lacking to date, but based on preliminary findings reported here further investigation of KIM-1 as a biomarker of KD in cats appears warranted.
References


Table 1. Primer sequences (5’ - 3’) for sequential amplification of the feline KIM-1 gene

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<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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90
Table 2. Signalment, laboratory data and KIM-1 immunostaining results

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<td>2</td>
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<td>-</td>
<td>-</td>
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<td>1</td>
<td>neg</td>
<td>Epithelial +</td>
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<td>85</td>
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<td>42</td>
<td>4.9</td>
<td>neg</td>
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1 Scale: neg to 3
2 RI 50-190umol/L
3 Blood urea nitrogen, RI 16-12mmol/L
4 Immunohistochemistry
5 Not performed
6 Post mortem examination
7 *Proteus*, *Streptococcus*
8 Immune-mediated hemolytic anemia
Fig 1. The full length feline KIM-1 cDNA isoform (A) consists of 894bp. Two shorter isoforms (B and C) of the gene are also expressed in adult kidney tissues, and consist of 810 and 705bp, respectively. There are 3 polymorphic sites that distinguish isoform C from A and B.
**Fig 2.** Schematic diagram showing the feline KIM-1 gene. Eight identified exons are represented as black boxes and spread over 21,059 bp of genomic DNA. A total of 3 expressed variants were identified in renal tissues consisting of 8 (isoform A), 7 (isoform B) and 6 (isoform C) exons, respectively.
Fig 3. Schematic of predicted functional domains of the feline KIM-1. Cysteine = ■
Potential glycosylation site = ■ Tyrosine kinase motif = ■
Fig 4. Phylogenetic tree of KIM-1 amino acid sequences from different species. Note feline and canine sequences are most closely related.
Fig 5. R-Renastick® KIM-1 urine immunoassay. Positive control reactions on left, test sample reactions on right. A. Test results from a cat that developed AKI during hospitalization. Initial test result was negative (top) and the subsequent test result was positive. B. Test results from a cat with acutely exacerbated chronic KD. Initial test results were positive and then became negative.
**Fig 6.** KIM-1 immunohistochemical staining of sections from cats with various kidney diseases: NovaRed substrate and hematoxylin counterstain. **A.** Cat with hypoperfusion, no prior kidney disease, positive KIM-1 urine, note tubular staining in the OSOM. Inset: Pre-incubation of antibody with KIM-1 peptide abrogates staining, 12.5x magnification. **B.** Cat with AKI due to bite wounds, sepsis and hypotension, positive urine KIM-1 result. Note staining of individual tubules. **C.** Cat with acute exacerbation of chronic renal disease. Renal fibrosis and tubular necrosis were present at post mortem. Urine KIM-1 result was positive on admission. There is sparse KIM-1 staining in individual proximal tubular cells and lumen. **D.** Cat with glomerulonephritis and interstitial nephritis due to chronic urolithiasis, negative urine KIM-1 result. Note absence of KIM-1 staining (B, C and D 100x magnification).
Fig 7. KIM-1 immunohistochemical staining of a kidney section (medullary ray) from a cat with an episode of hypoperfusion; NovaRed substrate and hematoxylin counterstain. **A.** Note variable staining among tubules (200x magnification). **B.** There is also variable staining among epithelial cells within a tubule (arrow, 400x magnification). **C.** Note KIM-1 negative tubular epithelial cells with intact brush border (arrows) indicating proximal tubule. Adjacent KIM-1 positive cells have indistinct brush borders and pyknotic nuclei (arrowhead) suggesting injury (1000x magnification).
Chapter 3

Characterization of kidney injury molecule-1 expression in normal and diseased feline kidney tissue

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Short title: KIM-1 expression in cat kidney

Keywords: Aquaporin-1, cat, serum creatinine, renal disease, vimentin, urinalysis

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Abstract

Kidney disease (KD) is common in older cats and presumed to arise from subclinical kidney injuries throughout life. Sensitive assays to detect kidney injury in cats are lacking. The sequence and structure of kidney injury molecule 1 (KIM-1), a sensitive and specific biomarker of kidney injury in humans and rodents, has been determined for the cat, and shedding into urine in naturally occurring acute kidney injury (AKI) was shown. In health, KIM-1 is rarely expressed in the proximal tubule of human and rodent kidneys, while in AKI expression of KIM-1 is markedly increased in the proximal tubule. The objectives of this study were to characterize KIM-1 expression in feline kidney sections by immunohistochemistry (IHC), and to correlate expression with markers of specific segments of the nephron and with morphologic changes indicative of AKI. Blood and kidney tissue samples were obtained from 8 cats without KD immediately prior to euthanasia, from 9 cats at variable time points after one hour of clamping of one renal artery, and from 9 cats with naturally occurring AKI and other concurrent illnesses. Expression of KIM-1 in proximal tubules of kidney sections, and correlation with expression of aquaporin-1 (AQP1) and vimentin, and with tubular injury, mitotic and inflammatory scores, was determined. Serum creatinine concentration (SCC) was measured in all cats, and analyzed in relation to KIM-1 expression in kidney sections. KIM-1 staining co-localized with AQP1 expression, and intensity of expression correlated with loss of brush borders, tubular necrosis and tubular regeneration. KIM-1 was transiently expressed in cortical proximal convoluted tubules 1 hour after ischemia. KIM-1 was highly expressed in the S3 segment of proximal tubules of cats with AKI from naturally occurring disease or induced by ischemia. S3 segments that were KIM-1
positive also expressed vimentin in cats with AKI, while vimentin staining in proximal tubules was rarely identified in sections from cats without KD. Vimentin expression increased over 12 days after experimental AKI, and was prominent in cats with naturally occurring AKI superimposed on chronic KD. SCC significantly increased after ischemia in individual cats with experimental AKI. In summary, findings indicate that expression of KIM-1 was specifically expressed in the S3 segment of the proximal tubule, and increased expression correlated with tubular injury. Detection of KIM-1 in kidney sections or urine samples may be a useful biomarker of AKI in cats.
Introduction

Chronic KD (CKD) is a common condition in older cats with an estimated incidence of 15 to 31%.\(^1\)-\(^3\) Large-scale studies to fully delineate the prevalence of CKD are lacking, and additionally cats may have clinically inapparent KD. The most common histopathological finding in cats with CKD is tubulointerstitial disease, which is likely a terminal change and not reflective of inciting causes.\(^4\)-\(^6\) While many studies have attempted to link disease processes to CKD, there are few identified definitive causes, and the pathogenesis of CKD remains largely unknown.

CKD is also common in humans, and recent epidemiologic studies suggest acute kidney injury (AKI) is a common precursor condition to CKD.\(^7\)-\(^9\) In human medicine, AKI has replaced the term acute renal failure, and encompasses the entire KD spectrum from minor reduction in glomerular filtration rate (GFR) to need for renal replacement therapy.\(^10\) AKI most often occurs concurrent with other diseases, but even modest or transient increases in serum creatinine concentration (SCC) are associated with increased mortality in people.\(^11\)-\(^13\) The Acute Kidney Injury Network (AKIN) criteria define AKI as a reduction in kidney function over 48 hours associated with an absolute SCC increase of \(\geq 26.4 \, \mu\text{mol/L} (\geq 0.3 \, \text{mg/dL})\) or a percent increase of \(\geq 50\%\) (1.5-fold from baseline), or a reduction in urine output.\(^14\) Thus, by these criteria, relative increases in SCC may be small and remain within reference intervals (RI) but nevertheless signify AKI. The discovery and application of new biomarkers to detect AKI in humans has further underscored the relative insensitivity of SCC for detection of early injury, but SCC nevertheless is still the most widely used biomarker for KD.\(^15\),\(^16\)
Little is known about the overall incidence of AKI in cats and its potential contribution to development of CKD. Some causes of fulminate AKI, such as urethral obstruction and ethylene glycol or *Lilium sp.* ingestion, are known, but in many cases the cause of AKI is unknown and may be multifactorial.\(^{17-21}\) Cats with AKI and modest increases in SCC are also at risk of increased mortality.\(^{22}\) It is plausible to surmise that in cats, as in people, episodic subclinical injury, or AKI with apparent recovery, progresses to CKD. It was suggested by the International Renal Interest Society (IRIS) to adopt a scoring system for AKI in animals that is similar to that in humans, based on intra-individual incremental increases in SCC regardless of RI (iris-kidney.com, accessed May 2014).\(^{23}\)

Interest in sensitive detection of AKI has lead to a search for serum and urinary biomarkers of AKI. One biomarker, kidney injury molecule-1 (KIM-1), has shown much promise in humans and rodents.\(^{24,25}\) KIM-1 is a renal tubular transmembrane glycoprotein thought to function in cell-to-cell or cell-to-matrix adhesion.\(^{24,25}\) KIM-1 has characteristics that make it a useful biomarker for AKI: low expression in healthy kidneys, rapid 3- to 100-fold increase after ischemic or toxic injury, and release of an extracellular portion into urine.\(^{24-28}\) Urine KIM-1 concentration correlated with severity of AKI, was increased prior to a rise in SCC, and decreased as kidney repair progressed.\(^{28}\) KIM-1 expression on immunohistochemistry (IHC) was specific to proximal renal tubules but variable in different segments depending on species and type of injury (toxic versus ischemic).\(^{26,29-32}\) In addition, regenerating tubules expressed both KIM-1 and vimentin, suggesting KIM-1 expression in tubular epithelium was associated with de-differentiation.\(^{24}\)
The feline KIM-1 gene and protein were recently described, and the extracellular domain was detected in the urine of critically ill but not clinically healthy cats using a urine immunoassay (see Chapter 2). KIM-1 immunopositive renal epithelium, believed to be the S3 (or straight) portion of the proximal tubule, was detected in tissues from cats with AKI, but neither precise location nor temporal expression was fully characterized. Knowledge regarding morphology, expression of specific markers, and function of different segments of the feline nephron is limited. Hence, the objectives of this study were: 1) To characterize KIM-1 expression in proximal tubules of cats without KD, cats with experimental AKI, and cats with naturally occurring AKI; 2) to evaluate KIM-1 expression over time in cats with experimental AKI; 3) to compare KIM-1, aquaporin-1 (AQP1) and vimentin expression in proximal tubules; and 4) to correlate histopathologic and SCC changes with KIM-1 expression in cats with and without AKI.

Materials and methods

Animals and procedures

Kidneys were obtained from 8 cats without clinical evidence of KD, based on urinalysis, SCC and urea concentration (4 neutered and 3 intact males, one intact female) that were 1-3 years of age and euthanized for reasons unrelated to this study (Institutional Animal Care Committee, protocol 10R030). Cats were sedated intramuscularly with ketamine (10 mg/kg) and medetomidine (20 µg/kg), or ketamine (10 mg/kg) and butorphanol (0.4 mg/kg) 15 minutes prior to collection of venous blood for measurement of SCC, and collection of urine by cystocentesis for urinalysis. Cats were then euthanized with an overdose of intravenous barbiturate; kidneys were removed immediately,
sagittally sectioned, fixed in 10% neutral-buffered formalin for 24-36 hours and processed routinely for histopathology.

Nine adult male intact cats, 15-29 months of age, that had been inoculated with *Brugia malayi* microfilaria but failed to become microfilaremic, were used in the experimental group (procedures approved by the Institutional Animal Care and Use Committee, University of Georgia). Food, but not water, was withdrawn 12 hours prior to the procedure. Cats were sedated with intramuscular acepromazine (0.01 mg/kg), buprenorphine (0.04 mg/kg), and ketamine (7 mg/kg), and maintained on isoflurane anesthesia via facemask. A midline laparotomy was performed, and the vasculature of the left kidney was isolated near the aorta and vena cava. A vascular clamp was placed across the left renal artery and vein for 60 minutes. Cortical wedge biopsies were obtained from the body of the left kidney immediately after onset of ischemia, and a second biopsy from the cranial pole one hour later just prior to release of the vascular clamp. Following release of the clamp, renal blood flow was confirmed by palpation of a pulse in the renal artery distal to the site of the clamp. Following 60 minutes of reperfusion, the arterial clamp was briefly applied again and a 3rd biopsy was obtained from the caudal pole of the left kidney. Hemostasis was confirmed prior to abdominal closure. Biopsy specimens were processed as above. Blood pressure was monitored and cats received intravenous fluids during and after surgery, and analgesia via transdermal fentanyl patch and transmucosal buprenorphine. SCC and urine specific gravity (USG) were measured at baseline and on post-operative days 1, 3, 6, and 12. Three cats were euthanized on each postoperative day 3, 6 and 12. On postmortem examination samples from control and ischemic kidneys were fixed in 10% neutral-buffered formalin and processed as above.
Nine cats admitted to the Health Sciences Centre at the University of Guelph either had cancer (2), CKD (3), cardiac arrest (1), sepsis (1), *Lilium sp.* toxicosis (1), or urethral obstruction (1). All cats developed AKI that did not improve with therapy either prior to admission or during hospitalization. These cats were euthanized due to progressive disease or untreatable other illness, and submitted for postmortem examination within 48 hours.

**Histopathology**

Sections of kidney were stained with hematoxylin and eosin (H&E) and by Periodic Acid–Schiff (PAS) reaction. Renal tubular segments, glomeruli, and the interstitium in the outer cortex and in the outer stripe of the outer medulla (OSOM) were identified on light microscopy according to morphologic features described by Maunsbach and Christensen, Christensen et al. and Ross et al. In sections from clinically healthy cats and cats with experimental AKI, injury in the proximal tubules was scored based on presence of nuclear pyknosis, attenuation, loss and coalescence of brush borders, cytoplasmic blebs, apoptotic cells or cells with cytoplasmic vacuoles containing KIM-1 immunopositive debris, cell loss into the tubular lumen, and evidence of cellular regeneration (cytoplasmic basophilia, flattening of tubules, anisokaryosis and nuclear or cell disarray) across the cortex and OSOM as described in prior studies. Scores based on whole tubules, or the portion of the tubule with change, ranged from 0 to 5 (0 = 0-1%; 1 = 2-10%; 2 = 11-25%; 3 = 26-50%; 4 = 51-75%; 5 = 76-100% of tubules affected). A score for proximal tubular injury was obtained by adding all separate scores. Inflammation was graded on a scale of 0 to 5 where 0 = ≤ 2; 1 = 3-10; 2 = 11-20; 3 = 21-
40; 4 = 41-60; 5 = >60 leukocytes in 10 fields of the cortex and OSOM at 400x magnification. Tubular luminal dilation was graded on a scale of 0 to 5 similar as tubular injury. The absolute number of mitoses in the cortex and OSOM was counted in 10 fields at 400x magnification.

Kidney sections from cats with naturally occurring AKI were variably affected by autolysis, therefore a more limited scoring scheme of morphologic change on a scale of 0 to 3 was applied with 0 = <1%; 1 = 2-25%; 2 = 26-50% and 3 = >50% of tissue affected.

**Immunohistochemistry**

Sections for IHC were deparaffinized in graded alcohol, rehydrated, and incubated consecutively for 10 minutes with endogenous enzyme blocker and 30 minutes with serum-free protein blocker (DakoCytomation, Mississauga, ON). For AQP1 staining, antigens were retrieved by heating to 125°C under pressure for 2 minutes and 45 seconds in pH 6.0 target retrieval solution (DakoCytomation). Anti-KIM-1 antibody (1:40 dilution, clone 219211, R&D Systems, Minneapolis, MN) and AQP1 rabbit polyclonal antibody (1:1200, EMD Millipore, Temecula, CA) were applied overnight at 4°C. Signal from bound antibodies was amplified with Envision Dual Link System-HRP (DakoCytomation), detected with Nova Red chromogen (Vector Laboratories, Burlington, ON), and slides were counterstained with hematoxylin. Antibodies to vimentin (1:200 dilution, clone V9) and pancytokeratin (1:100 dilution, clone AE1/AE3, both from DakoCytomation) were applied with an automated Decloaker chamber following the manufacturer’s instructions after antigen retrieval at 120°C, pH 6. Slides were batched in groups of 6-8 slides to ensure uniformity in processing. Each batch of slides included
negative (omission of primary antibody) control slides and multi-tissue block of positive control tissues. Additional controls for KIM-1 staining consisted of sections where antibody was pre-incubated for 2 hours with KIM-1 peptide (R&D Systems).

Immunohistochemical staining was localized based on tubular morphology (H&E and PAS stains), expression patterns of AQP1 in proximal tubules and loops of Henle of cats and dogs\textsuperscript{40} and expression of cytokeratin in collecting ducts and collecting tubules in cortex and OSOM.\textsuperscript{41} Serial sections were prepared for all IHC stains, identical regions were approximated for scoring and digital image capture, and cortex and OSOM were scored individually in cats without KD and cats with experimental AKI. One pathologist (JD) blinded to history assessed sections from cats with naturally occurring AKI.

**Distribution and intensity of immunohistochemical staining**

Stain location and intensity were scored in tissues from cats without clinical KD and with experimental AKI by one author (SKB) in a semi-blinded fashion. Both clamped and non-clamped kidney sections were on the same slide, precluding complete blinding, but cat and group identity were unknown. Scoring of KIM-1 IHC stain intensity was modified from Zhang et al.,\textsuperscript{29} to include assessment of cytoplasmic staining in tubular cells. Staining intensity was graded from 0 to 3 where 0 = no stain; 0.5 = weak fine granular staining focally along the luminal surface, pale cytoplasmic staining in non-atrophic proximal tubule cells; 1 = weak fine granular staining completely surrounding the luminal surface and in cytoplasm of non-atrophic proximal tubule cells; 2 = moderate granular staining completely surrounding the luminal surface, in cytoplasm of non-atrophic proximal tubule cells, and extending into inter-cellular junctions; and 3 = intense
granular staining completely surrounding the luminal surface, in cytoplasm of non-atrophic proximal tubule cells, and extending into intercellular junctions. IHC staining of vimentin was assessed on the proportion of proximal tubules with greater than or equal to 3 positive cells on a scale of 0 to 5 where 0-2%, 3-10%, 11-25%, 26-50%, 51-75%, 76-100% of a proximal tubule containing 3 or more cells with reactivity corresponded to increasing numerical scores. Staining for AQP1 and cytokeratin was not graded, but used to identify nephron segments.

**Grading of AKI**

Serum creatinine was measured with a Cobas 4800 biochemistry analyzer (Roche, Mississauga, ON). Urinalysis was performed using an automated urine dipstick reader and semi-quantitative microscopic sediment analysis. Specific gravity was determined with a veterinary refractometer. A grade of AKI was assigned in accordance with guidelines released by IRIS in December 2013 (Table 1).

**Data analysis**

All data analysis was performed with Prism 6.0 software (GraphPad, La Jolla, CA). Data from different groups were compared with paired or unpaired t-tests, and by correlation analysis. A two-way analysis of variance with repeated measures was used to evaluate the change in SCC over days in individual cats. Significance was set at \( p \leq 0.05 \).
Results

Animals

All cats without clinical KD had SCC within reference interval (SCC 42-125 μmol/L, RI 50-190 μmol/L) and adequate concentrating ability (urine specific gravity [USG] 1.039-1.064). Baseline SCC in cats with experimental AKI ranged from 70.2 to 159.1 μmol/L, and USG ranged from 1.040 to 1.060, which was also considered adequate. In cats with naturally occurring AKI, SCC on admission ranged from 126 to 1,703 μmol/L and USG ranged from 1.009 to 1.060.

Histopathology and IHC – Cats without KD

Nephron organization and terminology as applied in this chapter are schematically illustrated in Fig. 1. The proximal convoluted tubule consists of S1 and S2 segments, while the straight portion of the proximal tubule (S3 segment), thick ascending limb and collecting ducts collectively form the medullary ray within the inner cortex and the outer stripe of the outer medulla (OSOM). At 12.5x magnification, sections from a control cat illustrated superior contrast of PAS relative to H&E staining (Fig. 2A and B) in tubular segments. Vimentin IHC identified glomeruli, collecting tubules, and collecting ducts (Fig. 2C), while KIM-1 IHC faintly but specifically stained the S3 segment of proximal tubules (Fig. 2D). At higher magnification, PAS also better than H&E stain highlighted basement membranes and brush borders in sections of proximal tubules, and was therefore used throughout to detect loss of or changes in brush borders (Fig. 3). With PAS staining, cells of the S3 segment were identified by slightly columnar shape, basal location of large nuclei, light basophilic cytoplasmic staining, and lack of cytoplasmic
vacuoles. The cytoplasm of S1 segment cells stained slightly more magenta and had numerous large vacuoles, while S2 segment cells had more intense cytoplasmic staining and fine cytoplasmic vacuoles (Fig. 3). At higher magnification, S2 and S3 portions of tubules were distinguishable by less dense and shorter brush borders of S3 segment cells than of adjacent S2 segment cells (Fig. 4A and B). In addition, small PAS positive granules in the cytoplasm of S3 tubular cells but not S2 or S1 cells were particularly prominent in sections from control cats sedated with ketamine/medetomidine (Fig. 3A and B) and in sections of the non-clamped kidney of cats with experimental AKI (Fig. 4A and B).

KIM-1 IHC labeled the cytoplasm of cells in the S3 segment in sections from cats without clinical KD and in the non-clamped kidney of cats with experimental AKI (Fig. 1 and Fig. 4C). Expression was limited to S3 segment cells based on cell morphology, presence of a brush border stained with PAS, immuno-reactivity with AQP1 antibody, and lack of pancytokeratin (panCK) and vimentin expression (Fig. 4). Staining typically was faint and variably intense across the S3 segment, and not all S3 segments or cells were positive for KIM-1 (Fig. 5). All histopathologic and immunohistochemical features were systematically assessed (Table 2). In cats sedated with ketamine/medetomidine, the brush border of S3 but not S2 segments was uneven and ill defined, and there were numerous pyknotic nuclei at sites of disrupted brush borders (Fig. 3C and D). These features were not as prominent in cats sedated with ketamine/butorphanol. Differences were considered most likely to reflect different effects of sedation protocols on proximal tubules (p<0.01, Fig. 6A). KIM-1 positive cells often had loss of brush borders and
pyknotic nuclei, and relative expression of KIM-1 significantly correlated with injury scores (correlation coefficient $r = 0.55, p<0.01$; Fig. 6B).

AQPI positive staining was detected throughout all sections of the proximal tubular epithelium. In addition, loop of Henle, vasa recta, endothelium, and erythrocyte membranes were immunopositive, and faint glomerular staining was occasionally detected. In the S1 segment, staining was faint and generally confined to the luminal surface of cells with occasional fine granular staining at the basilar surface (Fig. 4D). In S2 segments, staining was more intense at the luminal surface than in S1, and there was often fine granular staining near the basilar cell membrane. S3 segments had intense staining along the luminal surface and all basolateral borders, creating a cobblestone effect, in addition to faint cytoplasmic staining. AQPI1 staining identified transitions from S2 to S3 segments corresponding to changes in brush border staining with PAS (Fig. 4B and D).

Most regions of the renal cortex and OSOM did not include panCK positive cells. A few individual cortical and OSOM tubule cells had positive staining corresponding to collecting ducts and collecting tubules. Occasional distal tubule cells had fine cytoplasmic granular staining. Expression of panCK in proximal tubules was not evident (*data not shown, see Appendix*).

Vimentin immunoreactivity was readily apparent in glomerular endothelium and in intensely stained cells of individual collecting tubules and ducts (Fig. 1C). In sections from cats without KD, rare cells of proximal tubules contained very fine cytoplasmic vimentin filaments, but otherwise immunoreactivity was absent in proximal tubules.
Histopathology and IHC – Cats with Experimental AKI

Cats with experimental AKI on day 3 had extensive tubular cell death, dilated tubular lumens, luminal cell casts, and inflammatory cells (neutrophils, eosinophils and lymphocytes) in dilated capillaries and the interstitial space of kidneys that had been clamped (Fig. 7). By day 6, dilation of individual tubular lumens remained pronounced, tubular epithelium was flattened, and there was a marked inflammatory cell infiltrate. In cats euthanized on day 12 after induction of experimental AKI, there was still marked interstitial inflammation in clamped kidneys, interstitial collagen deposition became apparent, and the number of tubules per field was decreased (Fig. 7). Changes in contralateral kidney sections were mild and consisted of mild brush border loss and moderate pyknosis.

KIM-1 expression was apparent in sections from the clamped kidney at each time point. Distribution across segments of the proximal tubule and within cells, and intensity of expression, were variable. Throughout all sections and time points, injury was consistently more severe in the OSOM than in the cortex (Table 3, Fig. 8). In biopsies obtained immediately after clamping, there was moderate staining of swollen cells in tubules that had indistinct vacuoles (Fig. 9A and B). Staining was most pronounced at the luminal aspect of cells. After one hour of clamping, cell swelling and dislodgement of cells into tubular lumens was more extensive, and in the corresponding areas of KIM-1 IHC sections, those areas stained intensely (Fig. 9C and D). Cell swelling was reduced, but there were more pyknotic nuclei, and luminal cells persisted one hour after removal of the clamp (Fig. 9E and F). At this time point, overall KIM-1 staining was less intense,
but dislodged cells in lumens were strongly immunoreactive, and there was positively stained fluid, likely regurgitated filtrate, in Bowman’s capsule.

In kidney sections from day 3, overall KIM-1 staining intensity was moderate, and involved predominantly S3 segments (Fig. 10A and B). There was variable staining among cells of the S3 segment, and luminal cell debris stained most intensely. Corresponding regions of the section stained for AQP1 confirmed that KIM-1 expression was in S3 segments, and outlined cellular debris in the tubular lumen (Fig. 10C and D). This finding is consistent with sloughing of tubular epithelial cells into the lumen and formation of urinary cell casts. Vimentin staining in matched sections yielded positive staining in the same tubules, indicating dedifferentiation, likely associated with repair of injured tubules (Fig. 10E and F). Not all cells of the same tubule stained equally, which suggests variable degrees of injury and repair. Similar findings of relatively uniform KIM-1 staining in S3 segments of non-clamped kidneys, and predominant staining of luminal cell debris in clamped kidneys, was noted in sections from another cat (Fig. 11A and B). Vimentin reactivity markedly differed between non-clamped and clamped kidneys (Fig. 11C and D). Injury as indicated by KIM-1 immunoreactivity on day 3 was similar in clamped kidneys from all cats (Fig. 12). At high magnification, intense KIM-1 staining of cytoplasmic fragments dislodged into tubular lumens was evident; nuclear and cellular debris in lumens stained positively, and remaining cells of the tubule had granular staining throughout the cytoplasm.

On day 6, non-clamped kidney sections had consistent, moderate KIM-1 staining of S3 segments (Fig. 13A). Tubular lumens were generally non-dilated, and cells were of similar size. Vimentin staining highlighted collecting tubules with low cuboidal
epithelium, and revealed sparse fine granular staining in S3 segment cells (Fig. 13B). Sections of the clamped kidney, however, showed many dilated tubule lumens that often contained intensely KIM-1-stained cellular debris (Fig. 13C). Corresponding vimentin-stained sections reveal extensive epithelial de-differentiation toward mesenchymal phenotype (Fig. 13D). Variable staining of tubules for KIM-1 was interpreted to reflect injury perpetuated from initial ischemia, subsequent reperfusion, influx of inflammatory cells (neutrophils, eosinophils and lymphocytes), and repair processes. Hence, not all tubules were expected to show an identical stage of injury and expression of KIM-1. PAS staining showed prominent large cytoplasmic globoid material in cells of the S3 segment, which was considered to reflect internalized brush border components and/or phagocytosed cell debris (Fig. 13E and F).

Sections from day 12 of the non-clamped kidney showed mild to moderate KIM-1 immunoreactivity throughout S3 segments of the proximal tubule, and faint expression in S1 and S2 segments (Fig. 14A). Vimentin immunoreactivity was more extensive than in sections of cats that did not undergo prolonged anesthesia (Fig. 2C), indicating that some tubules in non-clamped kidneys are de-differentiating. Sections from the clamped kidney had overall less KIM-1 immunoreactivity, greater inflammatory cell infiltrates, and very extensive vimentin staining (Fig. 14C and D). PAS staining highlighted a few remaining magenta cytoplasmic granules in cells of the S3 segment. These findings were interpreted to indicate that 12 days after ischemia and reperfusion, there was relative absence or loss of cells capable of expressing KIM-1, and extensive dedifferentiation. On the other hand, proximal tubules mildly injured from anesthetic agents and/or surgery-associated hypotension, remained capable of expressing KIM-1.
Comparison of injury scores relative to KIM-1 and vimentin IHC staining intensity, and to the number of mitoses, indicated significant correlations for each parameter at each time point except KIM-1 on day 6 and 12 (Fig. 15). Reasons for non-significant correlations were likely reduction in the number of cells capable of expressing KIM-1 on days 6 and 12. Inflammation was significantly greater in clamped than non-clamped kidneys on days 3 and 12, and in clamped kidneys on days 6 and 12 relative to day 3 (Fig. 16).

**Histopathology and IHC — Cats with naturally occurring AKI**

Cats with naturally occurring AKI had various illnesses leading to kidney injury (Table 4), and the time frame from euthanasia until tissue collection and fixation was variable. Accordingly, tissue preservation was inconsistent, and a more limited scoring scheme was applied. Albeit, AKI associated with an episode of sepsis 3 days prior to euthanasia induced KIM-1 expression in S3 segments (Fig. 17A), which was confirmed by AQP1 staining of cells in matching parallel sections (Fig. 17B). Widespread but variable vimentin reactivity highlighted that the injury was not acute, and may have occurred over several days (Fig. 17C). Similarly, in a cat treated with multi-agent chemotherapy for transitional cell carcinoma, AKI developed and was presumed to be a result of tumor lysis syndrome. Kidney sections showed dilated tubules with extensive loss of brush borders, moderate KIM-1 staining in AQP1 positive tubules, and relatively little vimentin staining in proximal tubules, consistent with injury prior to extensive de-differentiation (Fig. 18). SCC significantly correlated with kidney injury scores, but not with KIM-1 IHC staining intensity (Fig. 19). Likely reasons are that expression of KIM-1 is most
profoundly increased during acute injury, but SCC was generally measured several days after acute injury.

**Grading of AKI**

Experimentally induced AKI induced significantly increased SCC within individuals as assessed by repeated measures 2-way ANOVA ($p<0.01$), although mean SCC per group was not significantly different between days (Fig. 20). USG decreased initially, and then urine concentration increased slightly relative to day 0 (Fig. 21 and Table 5). Applying criteria of the AKI grading scheme of IRIS (Table 1), seven of nine cats with experimental AKI met IRIS criteria for AKI.

**Discussion**

In this study, tissues from cats without clinical KD and with a variety of causes of KD were assessed for expression of KIM-1. Expression localized to the S3 segment of the proximal tubule, and correlated with a unique pattern of AQP1 expression. Acute injury caused increased KIM-1 expression in the S3 segment. The feline nephron has segmental differences but only two segments, the convoluted (pars convoluta) and straight portion (pars recta) have been identified. These portions have been distinguished by presence of intracellular lipid droplets as detected with Sudan black and neutral lipid staining. PAS and alkaline phosphatase reactivity highlighted differences in the brush border that corresponded to segments identified with lipid staining. The straight portion of the proximal tubule (PT) has a unique complement of mitochondria, peroxisomes and endoplasmic reticulum that may impart greater susceptibility to injury.
from ischemia/reperfusion (I/R) and metabolic activation, while the convoluted portion may be more susceptible to injury from lysosomal overload. PAS brush border staining, cytoplasmic vacuolation, cellular arrangement, anatomic location, and AQP1 and KIM-1 IHC, identified three distinct segments of the PT in cats in this study. Morphologically and antigenically similar PT segments exist in rats, where the S3 segment corresponds to the straight portion, and the S1 and S2 to the convoluted portion of the tubule. Dogs have four proximal tubular segments, no distinct OSOM, and lipid vacuoles in the distal straight portion of the PT. 

Close approximation of serial kidney sections allowed assessment of morphologic features with different protocols. AQP1 IHC differential staining of each segment was retained through injury, which aided identification (Fig. 4). S3 segments had highest AQP1 staining along all cell membranes and the brush border, creating a cobblestone-like effect almost identical to that in humans, while S1 and S2 segments only had staining of the brush border. Variation in AQP1 staining along the PT in cats has previously been reported, but it was not explored to the same extent. In humans, KIM-1 co-localizes to cells expressing AQP1.

Cats with experimental AKI had marked tubular injury in the OSOM, and in particular in S3 segments. The injury was similar to that of experimental I/R in rats, where after 24 hours there was extensive brush border loss, tubular necrosis, epithelial flattening, tubular distension and luminal debris confined to the S3 segments of the OSOM and extending into the medullary ray. On days 3 and 6 post clamping, most cats still had evidence of ongoing tubular loss and necrosis. This is believed to result from inadequacy of blood re-flow and regional oxygenation differences in S3 segments,
and to account for ongoing injury\textsuperscript{36,51} More recent data suggest injury also induces expression of pro-inflammatory chemokines and cytokines such as interleukin (IL)-1, -6, and -8, tumor necrosis factor-\(\alpha\) and monocyte chemoattractant protein-1, which in turn amplify inflammation.\textsuperscript{52,53} Endothelial damage and dysfunction in the OSOM likely contribute to propagation of the inflammatory response, even after restoration of blood flow, resulting in continued ischemic injury and cell death. Damaged endothelial cells up-regulate intercellular adhesion molecule-1, and P- and E-selectin on their cell surface, leading to enhanced leukocyte adhesion and activation, which in turn perpetuates inflammation, worsens hypoxia and causes tubular obstruction.\textsuperscript{54} There is also distinct ‘zonation’ of blood vessels in the OSOM and inner stripe of the outer medulla, which may render cats more susceptible to continued injury after I/R than some other species.\textsuperscript{55,56}

In contrast to S3 segments, injury to S1 and S2 segments was mild to moderate and reversible, and there was little evidence of injury persisting beyond 3 days. This too is compatible with models of I/R in rodents, where S1/S2 brush border loss, progressive nuclear chromatin clumping, cell swelling and cell loss occur with ischemia, but during reperfusion within four hours there is brush border recycling and cell restoration.\textsuperscript{37,38,57}

The unique effect of different sedation protocols on S3 cells was unexpected. Sedation with ketamine/medetomidine resulted in greater injury and KIM-1 expression than ketamine/butorphanol. Medetomidine decreases cardiac output and increases vascular resistance in cats, and during the sedation period when neither blood pressure nor heart rate were monitored, this may affect renal perfusion.\textsuperscript{58,59} S3 changes noted may represent early apoptosis or necroptosis, but reversibility and long-term effect on renal
function are unknown.\textsuperscript{60,61} Tubular injury specific to particular sedative drugs might epitomize the relatively high susceptibility of cat kidneys to injury and subsequent development of CKD with progressive age.

Intensity of expression of KIM-1 correlated with tubular injury across all cats. Intraoperative biopsy samples clearly associated KIM-1 expression with injured cells in proximal cortical tubules (Fig. 9). During reperfusion, there was cellular recovery and decreased expression of KIM-1. Lack of persistent KIM-1 expression in S1/S2 proximal convoluted tubules in biopsies was likely related to rapid repair, but may also indicate functional differences between segments.

KIM-1 expression significantly correlated with tubular injury on day 3 but not days 6 or 12, despite some continued KIM-1 expression. Furthermore, in cats with naturally occurring AKI, KIM-1 was not associated with tubular injury. Possible reasons are that KIM-1 was down regulated as tubules repaired or de-differentiated, and then re-differentiated, and/or there was ongoing low-grade injury and therefore only limited expression of KIM-1. In addition, cats with naturally occurring AKI had various disease processes with injury that might have occurred days to weeks prior to euthanasia, and as a result kidneys had highly variable lengths of time in the reparative phase. A limitation of this study was that the full injury scoring system could not be applied to samples from cats with naturally occurring AKI, since tissues were not as well preserved precluding detailed assessment. Furthermore, experimental AKI likely induced most intense tubular expression of KIM-1 on the day of injury, but cats were not euthanized until day 3.

Vimentin is a cytoskeleton filament that is expressed in mesenchymal cells after
birth, but is re-expressed in injured epithelial cells. KIM-1 and vimentin were detected in injured and regenerating S3 segments of closely approximated regions in serial sections in all cats with AKI. This is in agreement with findings of Ichimura et al., where vimentin and KIM-1 were co-expressed in the same dedifferentiated cells undergoing regeneration after ischemia. Vimentin expression significantly correlated with tubular injury in experimental AKI on each day, indicating tubular injury persisted, or that tubular repair was incomplete by day 12.

Inflammation was significantly increased in clamped compared to contralateral non-clamped kidneys, and increased from day 3 to 12. The degree of inflammation was surprising. Inflammation was largely confined to the OSOM and to some degree the ISOM on day 3, but as days progressed inflammatory cells and interstitial cells were seen along the injured S3 tubules leading into the medullary rays of the cortex. Production of proinflammatory molecules by epithelium and endothelium is believed to be responsible for influx of inflammatory cells in I/R, and it may be that inflammation and incomplete repair are initials steps on the path toward development of CKD. Cats with experimental AKI were followed insufficiently long to assess if there was resolution of inflammation, but in one day 12 section there was marked tubular architectural change indicative of CKD.

KIM-1 staining intensity did not correlate with SCC in cats with naturally occurring AKI. This was most likely due to assessment of kidney tissues at highly variable intervals after AKI, and due to pre-existing CKD in at least three cats. In rodent experiments, KIM-1 is present in urine at the time of injury prior to increases in SCC, thus it is likely that KIM-1 tubular expression is highest at the time of injury and
Despite evidence of marked tubular damage, SCC increased prominently in only seven cats with experimental AKI. Differences in mean SCC between groups of cats were not significant, but within individual cats, there was a significant increase in SCC over time. This finding highlights need to consider change in SCC within individuals rather than within population-based reference limits. SCC is relatively consistent within an animal over time, but related to body mass, dietary protein concentration, age, drug therapy and others. Experimental cats in this study were male, young and intact, which may have contributed to relatively high SCC at baseline. Furthermore, these young cats may have had relatively high renal reserve minimizing SCC increases. Seven of nine cats had an increase in SCC of greater than 26.4 µmol/L, and in five cats this occurred within 48 hours of injury. Therefore, according to IRIS guidelines, these cats had AKI despite relatively modest increases in SCC. Of note, USG did not decrease, and even increased in some cats despite AKI. This is likely because there was no acute whole nephron loss at this time with experimental AKI, and because AQP1 expression persisted throughout the proximal tubule and loop of Henle. Furthermore, in cats AQP2 is expressed in collecting ducts, which may aid in maintaining concentrating ability despite injury in proximal tubules. In people, AQP1 may be up-reguated with injury to the proximal tubules, however, whether this occurs in cats is unknown.

There are limitations to this study. Firstly, the number of animals per group was limited. Findings were highly similar within experimental groups, and it would be difficult to justify using larger numbers of animals. Secondly, the time frame of the experimental study was limited to 12 days. This limitation did not allow assessment of progression of
AKI to CKD. Longer-term studies are needed to assess the risk for development of CKD, but evidence of AKI progressing to CKD in people, and the high degree of tubular change and inflammation in cats with experimental AKI, suggest such progression may occur in cats. Thirdly, only proximal tubules were examined due to focus of this study on KIM-1, but the other regions of tubules should also be assessed. Finally, post mortem timing limited preservation of tissue morphology in naturally occurring AKI, and underscored the importance of timely tissue procurement.

In conclusion, findings in this model of experimental AKI from I/R emphasize the need for more sensitive urine or serum biomarkers in cats. AKI, especially in animals with large renal reserve, currently cannot be detected. It is likely that many cats have undetected subclinical or episodic AKI, or AKI ascribed to insignificant prerenal azotemia, in association with sedation, anesthesia, surgery, cardiac disease, sepsis, trauma and other disease processes. As is illustrated in this study, small increases in SCC may be associated with a large degree of injury, inflammation and altered renal architecture. KIM-1 was expressed in S3 segments with mild injury, though unfortunately samples suitable for urine KIM-1 measurement were unavailable. Measuring KIM-1 in urine is a very promising marker of AKI in humans and rodents, and future studies will be directed at defining the utility of urine KIM-1 in cats. 24,26-28,68-70
References


**Figure 1.** Simplified nephron diagram showing tubular arrangement of the cortex and approximate division between the cortex, and outer and inner stripe of the medulla. OSOM = outer stripe of outer medulla, and ISOM = inner stripe of outer medulla. Small arrows indicate direction of tubular flow. Loops of Henle in the cats are long and extend into the inner medulla. For simplicity, not all vasculature is shown. Inset: Schematic outlining medullary ray. Adapted from Beeuwkes and Bonventre, Am J Physiol. 1975;229:695-713.
Figure 2. Kidney, cat without kidney disease, sedated with ketamine and medetomidine prior to euthanasia. Replicate sections of cortex and OSOM are closely matched. A. H&E stain. B. PAS stain. The light portion of the proximal tubule (arrow) corresponds to the KIM-1 positive S3 segment identified D (arrow). C. Vimentin IHC reactivity in glomeruli, collecting tubules (arrow) and ducts (arrowhead). D. KIM-1 IHC staining identifies S3 segments of proximal tubules within the medullary ray of the OSOM (arrow). All x12.5
Figure 3. Kidney, cat without kidney disease, PAS stain. A, B. Ketamine/butorphanol sedation. The brush border is mostly intact and of even height. Very few pyknotic nuclei in S3 or S2 tubules. C, D. Ketamine/medetomidine sedation. Note ill-defined and much reduced S3 brush border, but well-defined S2 brush border (arrows are at S2-S3 transition). There are numerous pyknotic nuclei in S3 segments at sites of disruption of the brush border. x200 A, C, x600 B, D
**Figure 4.** Kidney, cat, 3 days post experimental AKI, clamped kidney. **A.** H&E stain. Note S2 tubular cells contain small cytoplasmic vacuoles and are cuboidal while S3 tubular cells lack vacuoles and are slightly columnar. **B.** PAS stain. Note the cytoplasm of S2 cells stains deep magenta compared to cells in S3. Highly vacuolated cells are likely S1 segments. Arrow indicates area of transition from S2 to S3. **C.** KIM-1 light diffuse granular staining of cells in S3, and abrupt discontinuation of staining at the S2-S3 junction (arrows). **D.** AQP1 IHC. Note intense membranous and brush border staining in S3 cells. Arrows indicate S2-S3 transition. Note lack of membranous staining in S1 and S2. x400
Figure 5. Kidney, cat without kidney disease, sedated with ketamine and medetomidine, KIM-1 IHC. A. Specificity of KIM-1 immunoreactivity for S3 segments. Within S3 segments not all cells are immunoreactive and an adjacent S3 tubule does not stain (arrowhead). B. Many KIM-1 positive cells have pyknotic nuclei (arrow), but unstained cells do not have pyknotic nuclei and have full brush border (open arrow).
**Figure 6.** A. Tubular injury scores were significantly higher in kidney sections from cats with ketamine/medetomidine than ketamine/butorphanol sedation. Mean and standard deviation (SD) are shown. B. Relative expression of KIM-1 in S3 segments is significantly correlated with injury scores.
Figure 7. Kidney, cat, experimental AKI. Non-clamped (top row) and clamped (middle and bottom rows) kidney, H&E. Note marked increase in both interstitial cells and inflammatory cells (intra- and extravascular) in sections from clamped kidneys. There is loss of tubules and tubular architecture over time. Top x100, middle x200 and bottom x400
Figure 8. Clamping induces greater tubular injury in the OSOM than in the cortex at each of day 3, 6 and 12 (mean and SD, n=3 at each time point).
Figure 9. Kidney, cat, serial sections during and after experimental AKI. A, C and E are H&E stained; B, D and F are KIM-1 IHC. A and B are from biopsies obtained immediately after clamping of the renal artery. There is some cellular swelling and loss of whole cells into the lumen of several proximal tubules (arrow). There is light granular KIM-1 staining in many cells and of luminal debris. Glomeruli are negative. C and D are from biopsy samples obtained after 1 hour of clamping. Note marked cell swelling, necrosis and cell loss into the lumen of many proximal tubules * = tubular cast in lumen. D. KIM-1 staining is of highest intensity in cells dislodged into the tubular lumen. E and F are from biopsy samples obtained 1 hour after removal of the vascular clamp. Cells are less swollen, but individual epithelial cells persist in lumen (arrow). KIM-1 staining is overall reduced but cells in the lumen continue to stain positive (arrow). There is positively-stained extracellular fluid in Bowman’s capsule (filtrate regurgitation, open arrow). All x400.
Figure 10. Kidney, cat, 3 days after experimental AKI. A and B. KIM-1 IHC. Note difference in staining intensity among S3 tubules, which may indicate differing severity and timing of injury. Luminal cell debris stains intensely positive. C and D. AQP1 IHC. Intense membranous and brush border staining of S3 cells and of luminal cell debris. Arrow = area of S2-S3 transition; note lack of membranous staining in S2 cells. E and F. Vimentin IHC. Similar tubules stain positive for AQP1, KIM-1 and vimentin (arrowheads). Vimentin staining is variably intense among tubular cells. x200 A, C, E; x400 B, D, F
Figure 11. Kidney, cat, 3 days after experimental AKI. Non-clamped (A, C) and clamped (B, D) kidney. A. KIM-1 IHC: Cells in S3 segment stain lightly granular to moderately intense. There is flattening of the brush border (arrow) but no cell loss or pyknosis. Collecting duct, open arrow. B. KIM-1 IHC of clamped kidney shows positive tubules are dilated and filled with stained cellular debris. There is cell flattening, anisokaryosis, cell disorganization, apoptosis and pyknosis. C. Vimentin IHC shows lack of reactivity in S3 segment, but positive collecting duct (open arrow). D. Vimentin IHC shows extensive staining in tubules with most severe changes. All x400.
Figure 12. Kidney, cat, 3 days after experimental AKI. Sections from cat 1 (A, B) and cat 2 (C, D), clamped kidney. KIM-1 IHC. Note KIM-1 positive luminal debris, tubular dilation, cell rounding, loss of brush border, cytoplasmic blebs, single cell death, anisocytosis and anisokaryosis. Mitotic figure (B, arrow) and cell debris (D, arrowhead).
Figure 13. Kidney, cat, 6 days after experimental AKI. Non-clamped (A, B) and clamped (C, D, E, F) kidney. A. KIM-1 immunoreactivity in S3 segment is moderately intense. Rectangle = area enlarged in image B. B. Vimentin immunoreactivity is predominantly in collecting tubules, but individual proximal tubule cells also have fine granular staining (arrow) indicating a mild degree of dedifferentiation. C. KIM-1 staining is highly variable in the affected kidney. D. Vimentin IHC staining is more intense in tubules from clamped than non-clamped kidneys. E and F. There are cytoplasmic globoid inclusions in cells of S3 segments, PAS stain. x100 A, x200x B, x400x C, D, E, x600F.
Figure 14. Kidney, cat, 12 days after experimental AKI. Non-clamped (A, B) and clamped (C, D, E, F) kidney. A. KIM-1 immunoreactivity is most intense in S3 segments and faint in many S2 segments. Note glomeruli are unstained. B. Intense vimentin staining in glomeruli and collecting tubules, and faint staining in some S3 segments (arrows) suggesting sublethal injury to tubules. C. Faint KIM-1 staining in many tubules. There is marked loss of tubules, inflammatory infiltrate (*) and architectural rearrangement. D. Intense vimentin staining in tubules that are also KIM-1 positive. Vimentin staining is diffuse throughout tubules and also in interstitium. E and F. PAS stain shows fewer granular cytoplasmic inclusions and regenerating brush borders. x100 A, B; x200 C, D; x400 E, x600 F.
Figure 15. Kidney injury scores after experimental AKI are significantly correlated with expression of KIM-1 on day 3, with the number of mitotic figures on day 3 and 6, and with expression of vimentin on day 3, 6 and 12.
Figure 16. Kidney inflammation scores after experimental AKI are significantly higher on day 6 relative to day 3, and on day 12 relative to days 3 and 6 (* \( p < 0.001 \), mean and SD, \( n = 3 \) at each time point).
Figure 17. Kidney, cat, naturally occurring AKI due to sepsis 3 days prior to death. A. KIM-1 IHC shows marked variation in staining intensity between tubules, likely due to previous and ongoing injury. There is tubular dilation, flattening of cells and cell loss into the lumen. B. AQP1 IHC identifies KIM-1 positive tubules as S3 segments. C. Vimentin IHC staining is widespread, granular and of variable intensity. KIM-1 positive tubules are also vimentin positive. Intensely stained tubules with low epithelium (arrows) are collecting ducts. Overall staining properties and cell damage are very similar to that of experimental AKI. All x100.
Figure 18. Kidney, cat, naturally occurring AKI due to tumor lysis. A. PAS stain shows extensive loss of brush border. B. KIM-1 IHC indicates moderately intense staining of S3 segments. C. AQPI staining intensity confirms KIM-1 positive tubules as S3 segment. D. Vimentin IHC staining indicates limited reactivity.
Figure 19. In cats with naturally occurring AKI, SCC is significantly correlated with kidney injury scores but not with KIM-1 immunoreactivity (n=9).
Figure 20. SCC increases after experimental AKI, but means between groups are not significantly different (mean and SD, n=3 at each time point).
Figure 21. Experimental AKI induces a significant increase in USG on day 6 (all other comparisons $p>0.05$; $n=6$ on day 0, 1, 3 and 6; $n=3$ on day 12).
<table>
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<th>AKI Grade</th>
<th>SCCa</th>
<th>Clinical description</th>
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| I         | <140 μmol/L (≤1.6 mg/dL) | Non-azotemic AKI:  
    a. Documented AKI (historical, clinical, laboratory, or imaging evidence of acute kidney injury, clinical oliguria/anuria, volume responsiveness) and/or  
    b. Progressive non-azotemic increase in SCC; ≥26.4 μmol/L (≥0.3 mg/dL) within 48 hours  
    c. Measured oliguria (<1 ml/kg/hr) or anuria over 6 hrs |
| II        | 141 – 220 μmol/L (1.7 – 2.5 mg/dL) | Mild AKI:  
    a. Documented AKI and static or progressive azotemia  
    b. Progressive azotemic increase in SCC; ≥26.4 μmol/L (≥0.3 mg/dL) within 48 hours, or volume responsivenessb  
    c. Measured oliguria (<1 ml/kg/hr) or anuria over 6 hrs |
| III       | 221 – 439 μmol/L (2.6 – 5.0 mg/dL) | Moderate to severe AKI:  
    a. Documented AKI and increasing severities of azotemia and functional renal failure |
| IV        | 440 – 880 μmol/L (5.1 – 10.0 mg/dL) |  |
| V         | >880 μmol/L (>10.0 mg/dL) |  |

a Serum creatinine concentration  
b Volume responsiveness = increase in urine production to >1ml/k/hr over 6 hrs; and or decrease in SCC to baseline over 48 hrs
Table 2. Lesion and immunohistochemical scores of kidney sections from cats without kidney disease

<table>
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<tr>
<th>Sedation</th>
<th>Region</th>
<th>Pyknotosis(^a)</th>
<th>Brush border(^a)</th>
<th>Cytoplasmic blebs(^b)</th>
<th>Apop.(^c)</th>
<th>Cell loss(^c)</th>
<th>Cell repair(^c)</th>
<th>Tubular injury score</th>
<th>Tubular dilation</th>
<th>Total injury score</th>
<th>Mito(^e)</th>
<th>KIM-1 IHC(^h)</th>
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\(^a\) Proportion of tubules with change: 0 = 0-2%; 1 = 3-10%; 2 = 11-25%; 3 = 26-50%; 4 = 51-75%; 5 = >75%

\(^b\) Attenuation, loss and coalescence of brush border

\(^c\) Apoptosis consisting of nuclear condensation, cytoplasmic vacuolation with KIM-1\(^-\) debris

\(^d\) Cytoplasmic basophilia, anisokaryosis, cellular disorganization

\(^e\) Proximal convoluted tubules/Distal tubules/Collecting tubules and ducts

\(^f\) Inflammation, leukocytes in interstitial: 0 = none; 1 = 1-4/40x; 2 = 5-30/40x; 3 = 31-100/40x; 4 = >100/40x

\(^g\) Mitosis 10 fields at 400x magnification

\(^h\) 0 = no staining; 1 = faint granular staining along part of luminal surface and in cytoplasm; 2 = prominent granular staining along entire luminal and cytoplasm and intercellular junctions; 3 = intense large granular staining along luminal surface, and in cytoplasm and intercellular junctions

\(^i\) Proportion of tubules with \(\geq 3\) vimentin positive cells: 0 = none; 1 = <10%; 2 = 10-25%; 3 = 26-50%; 4 = 51-75%; 5 = >75%

\(^j\) Outer stripe of the outer medulla
Table 3. Lesion and IHC scores of kidney sections from cats with experimental AKI

<table>
<thead>
<tr>
<th>Euthanasia Kidney</th>
<th>Region</th>
<th>Pyknosis*</th>
<th>Brush border**</th>
<th>Cytoplasmic blebs*</th>
<th>Apop. **</th>
<th>Cell loss*</th>
<th>Cell repair ***</th>
<th>Tubular injury score</th>
<th>Tubular dilation PCT*** DT/CD**</th>
<th>Total injury score</th>
<th>Infl.†</th>
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*Proportion of tubules with change: 0 = 0-2%; 1 = 3-10%; 2 = 11-25%; 3 = 26-50%; 4 = 51-75%; 5 = >75%*

*Attenuation, loss or coalescence of brush border*

*Apoptosis consisting of nuclear condensation, cytoplasmic vacuolation with KIM-1+ debris*

*Cytoplasmic basophilia, anisokaryosis, cellular disorganization*

*Proximal convoluted tubules/Distal tubules/Collecting tubules and ducts*

*Inflammation, leukocytes in interstitium; 0 = <2/40x; 1 = 3-10/40x; 2 = 11-20/40x; 3 = 21-40/40x; 4 = 41-60/40x field*

*Mitosis 10 fields at 400x magnification*

*0 = no staining; 1 = faint granular staining along part of luminal surface and in cytoplasm; 2 = prominent granular staining along entire luminal surface and in cytoplasm and intercellular junctions; 3 = intense large granular staining along luminal surface and in cytoplasm and intercellular junctions*

*Vimentin, proportion of tubules with ≥3 vimentin positive cells: 0 = rare or none; 1 = <10%; 2 = 10-25%; 3 = 26-50%; 4 = 51-75%; 5 = >75%*

*Serum creatinine concentration (μmol/L) increase from day 0*

*Outer stripe of the outer medulla*

*Ischemia reperfusion*

*Not done*
Table 4. Lesion and immunohistochemical scores of kidney sections from cat with naturally occurring AKI

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<th>Fibr.</th>
<th>Casts</th>
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<th>VIM. IHC</th>
<th>SCC</th>
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- **Glomerular lesions**
- **Tubular necrosis**
- **Inflammation**
- **Fibrosis**
- **Vimentin**, proportion of tubules with ≥3 vimentin positive cells: 0 = rare or none; 1 = &lt;10%; 2 = 10-25%; 3 = 25-50%; 4 = 51-75%; 5 = &gt;75%

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- **Serum creatinine concentration during hospitalization**
- **Outer stripe of outer medulla**
### Table 5. Laboratory data in cats with experimental AKI

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<th>USG&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup> Serum creatinine concentration
<sup>b</sup> Urine specific gravity
Chapter 4

Summary and Implications of Research

The research presented here was aimed at investigating whether KIM-1 would be a useful marker of acute kidney injury (AKI) in cats. During the investigation addressing this question, many areas of uncertainty in the pathogenesis of kidney disease (KD) became apparent. Initially, feline KIM-1 gene and cDNA structure and sequence were determined, and the KIM-1 extracellular domain was detected in urine of cats with presumed AKI but not healthy cats. Next, focus was on understanding and localizing KIM-1 expression in healthy and diseased kidney tissue by immunohistochemistry (IHC). At this point it then became clear that neither segmental variation of the feline proximal tubule nor the pathophysiology of AKI or chronic kidney disease (CKD) were understood. Furthermore, the relationship between the magnitude of cellular change and tubular injury with serum creatinine concentration (SCC) was ambiguous. In many cats with clinical and laboratory evidence of severe KD, routine histopathologic assessment of kidneys indicated absence of lesions or only mild changes. Furthermore, it became evident that tissues from cats processed immediately had strikingly superior architectural and cellular preservation relative to samples from cats where post-mortem tissue fixation was delayed. In the latter samples, changes in brush border, basement membrane and cell morphology were reasonably discernable with periodic acid Schiff (PAS) but not hematoxylin and eosin (H&E) stains. Hence, it was concluded that in order to improve histopathologic evaluation of KD, and to more sensitively detect functional impairment, it is essential that 1) kidney tissues are fixed and processed rapidly following
procurement or euthanasia; 2) sections are routinely stained with PAS in addition to H&E; and 3) all components of the nephron, interstitium and lumen are systematically assessed.

Comparison of different IHC stains to localize KIM-1 allowed differentiation of three segments of the proximal tubule based on staining characteristics, morphology and anatomic location. Aquaporin (AQP1) expression was readily detected in injured and non-injured proximal tubules, and yielded three very distinct staining patterns that allowed identification even of severely injured S3 segments. AQP1 staining appeared more intense in clamped kidneys than in contralateral non-clamped kidneys, which may indicate that AQP-1 is upregulated after injury. This hypothesis remains to be investigated, but might account for highly concentrated urine noted in some cats with AKI or CKD. Electron microscopy would be helpful to determine whether ultrastructural features distinguish segments of the proximal convoluted tubule.

CKD occurs more often in cats than dogs, and the location of KD lesions differs. Dogs more commonly have more glomerular disease and cats more commonly tubular disease. Differences in tubular and vascular arrangement in the OSOM likely contribute to this differential susceptibility, with cat kidneys being more sensitive to changes in blood flow.

A more sensitive test than SCC is needed to detect AKI, and interpretation of SCC needs to be changed to maximize sensitivity for reduced glomerular filtration and tubular function. As illustrated in this study, small changes in SCC can indicate severe injury regardless of absolute values remaining in reference interval (RI).
Recommendations by the International Renal Interest Society (IRIS) enhance utilization of SCC for detection of AKI, may still be insufficiently sensitive.

A feline specific KIM-1 urine immunoassay would likely be able to detect AKI before increase in SCC, as it does in the rat and human, but this remains to be determined. At this point the initiation, duration and magnitude of feline KIM-1 shedding into urine are unknown. All likely depend on the nature, extent and duration of injury. A urine KIM-1 immunoassay may be most useful in hospital settings to detect AKI associated with sedation, anesthesia, drug or chemotherapy, hypotension, sepsis, and critical injury. The utility of detecting KIM-1 by IHC for purposes other than research remains to be determined. In vivo collection of kidney samples, such as those obtained with ‘Tru-cut’ biopsies, rarely include S3 segments of the OSOM, and may therefore preclude detecting areas of greatest injury from hypoperfusion or oxidative damage. For these reasons generation of a cat-specific urine immunoassay for KIM-1 appears to be the most promising route toward better detection of AKI.
APPENDIX

GenBank Submission Information

KF540032.1 Felis catus hepatitis A virus cellular receptor 1 variant A (HAVCR1) mRNA, complete cds, alternatively spliced

KF540033.1 Felis catus hepatitis A virus cellular receptor 1 variant B (HAVCR1) mRNA, complete cds, alternatively spliced

KF540034.1 Felis catus hepatitis A virus cellular receptor 1 variant C (HAVCR1) mRNA, complete cds, alternatively spliced
<table>
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<tr>
<th><strong>Antigen</strong></th>
<th><strong>Vendor</strong></th>
<th><strong>AB source</strong></th>
<th><strong>Clone/number Platform</strong></th>
<th><strong>Dilution</strong></th>
<th><strong>Antigen retrieval</strong></th>
<th><strong>Detection system</strong></th>
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<tr>
<td>Cytokeratin 7</td>
<td>Dako</td>
<td>mouse Mab(^1)</td>
<td>OV-TL 12/30</td>
<td>Ventana</td>
<td>1/50, 60m</td>
<td>PK(^2) (12m)</td>
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<td>AE1/AE3</td>
<td>Dako</td>
<td>1/100, 30m</td>
<td>HIER pH 6(^4)</td>
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<td>Dako</td>
<td>mouse Mab</td>
<td>V9</td>
<td>Dako</td>
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\(^1\)Monoclonal antibody
\(^2\)Proteinase K
\(^3\)High molecular weight
\(^4\)Heat induced epitope retrieval pH6 (citrate)-decooker
\(^5\)Glutathione-S-transferase α
\(^6\)overnight
\(^7\)Aquaporin-1
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<th>Vimentin</th>
<th>Pancytokeratin</th>
<th>CK&lt;sup&gt;3&lt;/sup&gt; 7</th>
<th>CK 20</th>
<th>HMW&lt;sup&gt;4&lt;/sup&gt; CK</th>
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<td>-</td>
<td>-/+ luminal memb</td>
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<tr>
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<tr>
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<sup>1</sup> Aquaporin-1  
<sup>2</sup> Glutathione S-transferase α  
<sup>3</sup> Cytokeratin  
<sup>4</sup> High molecular weight  
<sup>5</sup> Variable cell membrane and cytoplasm staining intensity between cells within the same tubule  
<sup>6</sup> Inconclusive  
<sup>7</sup> Cytoplasmic  
<sup>8</sup> Membranous  
<sup>9</sup> Inner stripe of outer medulla  
<sup>10</sup> Outer strip of outer medulla  
<sup>11</sup> Not examined

It should be noted that cytokeratin 7, cytokeratin 20 and high molecular weight cytokeratin were not fully optimized for the cat kidney. There was background staining in the outer cortex of CK 7 and HMW CK, therefore IHC results should not be regarded as accurate.
**Figure 1.** Kidney, cat without clinical kidney disease. 

**A & B.** GSTα immunoreactivity is restricted to in the proximal and distal tubules in the cortex, outer stripe of the medulla (OSOM) and straight portion of the distal tubules in the inner stripe of the outer medulla (ISOM). No GSTα immunoreactivity is present in the inner medulla.  

**C.** GSTα immunoreactivity is confined to the cytoplasm of the proximal (PT) and distal tubules (DT) of the cortex. The distal tubules have a granular cytoplasmic pattern as compared to the proximal tubules.  

**D.** GSTα immunoreactivity in straight portion of the distal tubules in the inner stripe of the outer medulla (ISOM). x100 A, B, x400 C, x200 D.
Figure 2. Kidney, cat without clinical kidney disease. A. Pancytokeratin immunoreactivity within collecting ducts and tubules of the cortex, outer stripe of outer medulla (OSOM) and collecting ducts of the inner stripe of outer medulla (ISOM), and the medulla. B and C (inset of B). At higher magnification variable luminal membranous immunoreactivity and slight cytoplasmic immunoreactivity of the straight distal tubules of the OSOM and ISOM can be seen (arrows). Individual cells of the collecting ducts have intense membranous and cytoplasmic staining (arrowheads) while other cells have only cytoplasmic staining. x40 A. x200 B. x400 C.
Figure 3. Kidney, cat without clinical kidney disease. A. Cytokeratin 7 faint cytoplasmic immunoreactivity in proximal tubules and straight distal tubules in the outer and inner stripe of the outer medulla (OSOM and ISOM, respectively). B. Inset of A, specific cytoplasmic staining of straight distal tubules (ascending thick limb of Loop of Henle) can be seen. No other tubules are immunoreactive. x100 A. x400 B.