CHRONIC KIDNEY DISEASE – MECHANISMS OF PROGRESSION IN COMPANION ANIMALS

by

BIANCA NATÁLIA FERREIRA DE MOURA LOURENÇO

(Under the Direction of Amanda E. Coleman)

ABSTRACT

Chronic kidney disease (CKD), a prevalent metabolic disorder in aged companion animals, is irreversible and progressive. Proteinuria, chronic renal hypoxia, and maladaptive stimulation of the renin-angiotensin-aldosterone system (RAAS) are among the most widely cited mechanisms for the intrinsic progression of CKD. Nevertheless, significant knowledge gaps exist in the role of these factors in canine and feline CKD.

Glomerular disease, a common cause of canine CKD, is classically associated with proteinuria, the magnitude of which is positively correlated with hastened progression of CKD, increased risk for uremic crises, and renal and all-cause mortality. Antagonism of the RAAS is a crucial part of antiproteinuric therapy; however, angiotensin-converting enzyme inhibitors (ACEi) are not universally efficacious. In a prospective, double-masked, randomized clinical trial that evaluated dogs with persistent renal proteinuria, we showed that compared to an ACEi, the angiotensin receptor blocker telmisartan led to greater reduction of proteinuria, offering promise as a first-line therapy.

In contrast to dogs, the majority of cats with CKD are affected by primary lesions of the tubulointerstitial compartment, with fibrosis and inflammation developing from the early stages.

Chronic renal hypoxia is a described cause and consequence of tubulointerstitial fibrosis, activating numerous pro-inflammatory and pro-fibrotic pathways. Our work demonstrates upregulation in the transcription of specific fibrosis mediators, including members of the matrix metalloproteinase family, hypoxia-inducible factor and transforming growth factor, in both ischemia-induced and naturally-occurring feline CKD.

While inappropriate activation of the RAAS is well-defined in CKD of many non-feline species, data from cats offer conflicting results. Here, we not only document upregulation of intrarenal renin-angiotensin system (RAS) components in experimentally-induced and naturally-occurring feline CKD, but also show that markers of the circulating RAAS are inconsistently correlated with those of the intrarenal RAS in feline CKD models and healthy cats. Therefore, as in other mammals, regulation of tissular RAS appears to be independent of that of its circulating counterpart.

Collectively, our studies provide valuable evidence to guide refinement of antiproteinuric therapy in dogs, and advance our knowledge of the pathophysiology underlying feline CKD progression, identifying new candidate biomarkers and therapeutic targets for renal disease in cats.

INDEX WORDS: Canine, Feline, Chronic Kidney Disease, Proteinuria, Hypoxia, Ischemia, Fibrosis, Renin, Angiotensin, Aldosterone

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DEDICATION

To my mother, Natália Ferreira, for the unconditional emotional support every step of the way; to my father, Carlos Lourenço, for teaching me to be curious and inquisitive, and instilling in me the love for science; to my sister, Mariana Lourenço, the damsel in distress and object of adoration of this "love monster", for coming along and challenging me from her first week of life. I have missed each of you every single day of this journey.

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CHAPTER 1

INTRODUCTION

Chronic kidney disease (CKD), defined as an alteration of the renal structure or function that persists for greater than three months,[1, 2] is a leading metabolic disorder in companion animals, particularly those of advanced age.[3] Estimates of disease prevalence in domesticated cats and dogs vary among publications, each examining different populations and using distinct study methodologies. Early feline studies reported an overall prevalence ranging from 1 to 3% of all cats presented to both private practice[4] and teaching hospital[5] settings. These data are corroborated by more recent publications reporting overall prevalence estimates ranging between 1.2 and 3.6% in cats attending primary care practices in the United Kingdom.[6, 7] When only geriatric cats are considered, prevalence estimates increase to 31% in cats older than 15 years of age,[5] with more current prevalence estimates approaching 50-86% in certain geriatric feline populations.[8] A comparable pattern is observed in dogs, for which more dated literature estimates the prevalence of CKD as 0.5 to 1.5% in a general canine population, and 10% in a geriatric referral population.[9] Similarly, a more recent study of a large population of dogs attending veterinary practices in the United Kingdom reported a prevalence of 0.02% to 1.44%, with dogs greater than 12 years of age having approximately 5.5 times greater odds of being affected by CKD when compared to dogs aged 7 to 12 years.[10]

The specific primary causes of CKD are diverse. However, regardless of the inciting cause, CKD is irreversible and slowly progressive.[3] Because the kidneys have numerous functions with a profound impact on whole-body homeostasis and regulation of other organ

systems, decreased renal function may be associated with systemic clinical signs that greatly impact the individual's quality of life. Accordingly, renal disease has a significant impact on longevity, with renal disorders representing the most common cause of death in cats older than 5 years of age.[11] Given its substantial burden on animal welfare and longevity, CKD has garnered the attention of the scientific community for several decades. In recent years, the development of novel biomarkers, which allow earlier diagnosis[12, 13] and offer the opportunity for timelier therapeutic intervention, has heightened this attention.

Current CKD treatment strategies focus on delaying progression of the disease and ameliorating its clinical signs.[3, 14] Numerous factors favoring the progression of CKD have been broadly described; these include systemic and glomerular hypertension, activation of the renin–angiotensin–aldosterone system (RAAS), stimulation of other specific cytokines and growth factors [e.g., transforming growth factor (TGF)-β], podocyte loss, proteinuria, hyperphosphatemia with associated CKD-bone mineral disorder, chronic renal hypoxia, and tubulointerstitial fibrosis.[15-17] Presently, interventions directed at reducing the rate of intrinsic progression and minimizing the complications of CKD in companion animals are limited to the reduction of phosphate intake and to the management of proteinuria, hypertension, anemia, dehydration, and electrolyte and acid base abnormalities that often accompany reduced renal function.[3] A better understanding of the mechanisms perpetuating renal damage in CKD would not only facilitate the discovery of new diagnostic tests, and allow for earlier disease detection, but also identify candidate targets for directed therapies aimed at halting disease progression. Furthermore, because CKD is a major worldwide public health concern, affecting approximately 10% of human beings aged ≥ 20 years,[18] the benefits of understanding of these mechanisms

would extend beyond the confines of veterinary medicine, providing important translational opportunities.[19]

Research objectives

The main research objectives of this work were:

1. To evaluate the efficacy of the RAAS antagonist, telmisartan, for the treatment of persistent, pathologic, renal proteinuria in dogs with CKD;
2. to characterize the transcription of genes involved in molecular pathways associated with renal fibrosis in cats with experimentally-induced and naturally-occurring CKD; and
3. to characterize the intrarenal renin-angiotensin system (RAS) and circulating RAAS in cats with experimentally-induced and naturally-occurring CKD.

CHAPTER 2

LITERATURE REVIEW

The term "chronic kidney disease" describes a myriad of conditions that persistently affect the function and/or structure of the kidneys. CKD may be initiated by a variety of familial, congenital, or acquired disorders. In human beings, the causes of CKD vary according to factors such as race, country, and economic status.[20] Diabetes and hypertension are leading causes of human CKD in developed countries, whereas glomerulonephritis and unknown causes are more common in Asia and sub-Saharan Africa.[21] Not surprisingly, the major inciting causes for this disease differ among cats, dogs and human beings. However, its irreversible and progressive course, as well as many of the described mechanisms of progression of CKD, are likely shared by these species.[22, 23]

The kidneys from patients with end-stage renal disease (ESRD) almost invariably demonstrate glomerulosclerosis and tubulointerstitial fibrosis,[24, 25] suggesting a common final pathway of progressive injury.[15] Due to its complex pathophysiology, the molecular basis of disease progression remains poorly understood. The study of mechanisms of progression of CKD has utilized several experimental models of renal deterioration, including the instrumental remnant kidney model. Work performed in these models has shown that reducing the number of functioning nephrons triggers a sequence of events that results in compensatory growth of the remaining nephrons, as well as functional adaptations, which include increases in blood flow and intracapillary glomerular pressure, increased single-nephron filtration rate, and net reabsorption of solutes and water.[26, 27] Early in the disease course, these adaptations are able to partially

compensate for nephron loss; however, they eventually become maladaptive, leading to further nephron injury, and perpetuating a vicious cycle that ultimately results in the progressive kidney disease.[28, 29] In addition to these hemodynamic adaptations, the development of systemic hypertension, proteinuria, chronic renal hypoxia, and activation of various cytokines, growth factors and hormones - including those of the RAAS - also contribute to self-perpetuating renal damage.[15, 29-32]

The renin-angiotensin-aldosterone system

Working closely with the sympathetic nervous system, the RAAS is a major regulator of systemic arterial blood pressure (BP) and fluid and electrolyte balance. In the "classical view" of this cascade, the RAAS is seen as a circulating, endocrine system with angiotensinogen (AGT; an α_2-globulin produced by the liver) being cleaved by renin (the rate-limiting enzyme, released from renal juxtaglomerular cells into the systemic circulation) to generate the decapeptide angiotensin I (AngI; also known as angiotensin [1-10]). Angiotensin I is, in turn, cleaved by angiotensin-converting enzyme (ACE; principally bound to the surface of the vascular endothelium) into the biologically active end-product of the system, the octapeptide angiotensin II (AngII; also known as angiotensin [1-8]).[33, 34] Predominantly through interaction with the angiotensin II type 1 receptor (AT_1R), AngII initiates its most well-known effects, which include vascular smooth muscle contraction, aldosterone secretion, dipsogenic responses, renal sodium reabsorption, pressor and tachycardic responses, and inhibition of renin release.[35] The net effect of these responses is conservation of salt and circulating blood volume, and maintenance of normal BP. Angiotensin II's BP-increasing effects are due not only to its role as a potent peripheral vasoconstrictor, but also to its actions on the renal – particularly glomerular – hemodynamics. In the renal

microvasculature, AngII causes constriction of both the afferent and efferent arterioles of the glomerulus, but its effect is more pronounced on the efferent vessels.[36] By preferentially constricting the efferent arterioles, AngII reduces renal blood flow while increasing glomerular filtration through an increase in glomerular capillary pressure.[36] These hemodynamic changes increase filtration fraction, thereby increasing oncotic pressure within the glomerular capillary, while simultaneously decreasing hydrostatic pressure in the downstream peritubular blood vessels.[37] The reduction in peritubular capillary pressures promote sodium and water reabsorption from the proximal tubule into the interstitium, and act in conjunction with AngII's direct stimulation of sodium and water reabsorption in the renal tubules.[37]

Since its first description in 1896, the traditional view of the RAAS has been significantly updated and expanded to include new enzymatic pathways, receptors and peptides, as well as the presence of local, or tissular RASs.[38, 39] Our understanding of this system has become increasingly complex with the description of alternative ACE-independent pathways for AngII generation (e.g., chymase), a second form of ACE (ACE2), additional AngII receptor types (e.g., AT_2R, AT_3R and AT_4R), and the discovery of biologic actions of the (pro)renin receptor and angiotensin peptides other than AngII.[35, 39, 40]

Besides its effects on fluid and electrolyte balance, AngII also induces the expression and/or transcription of growth factors and proliferative cytokines, including TGF-β1, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), platelet-derived growth factor and nuclear factor-κB, and perpetuates the production of nephrotoxic reactive oxygen species, leading to inflammation, fibroblast formation, and collagen deposition.[36, 37, 40, 41] The pro-fibrotic and pro-inflammatory effects of AngII, which is now recognized as a morphogenic cytokine, particularly in the context of renal disease, are primarily mediated via the AT_1R.[40]

Conversely, the AngII type 2 receptor (AT_2R) mediates opposing effects (e.g., systemic and renal vasodilatation, decreased renal sodium reabsorption, nitric oxide release, and inhibition of proliferation, growth and fibrosis), counterbalancing those effected by the AT_1R.[36, 39]

While renin and AGT have relatively long plasma half-lives, AngII is rapidly degraded by aminopeptidases, known as angiotensinases, to form angiotensin III (AngIII or angiotensin [2-8]), angiotensin (1–7), and angiotensin IV (AngIV or angiotensin [3–8]).[38] These smaller angiotensin peptides are biologically active, although their plasma concentrations are much lower than those of AngII.[42] Like AngII, AngIII is also capable of interacting with both the AT_1R and AT_2R,[39] though it appears to have greater selectivity for the AT_2R.[43] AngIV may be generated from AngIII and interacts with the AT_4R, which has functions associated with cognition (e.g., memory acquisition and recall), regulation of blood flow, inhibition of renal tubular sodium reabsorption, and cardiac hypertrophy.[35] Ang (1-7) can be formed through the action of ACE2 on AngII and appears to have actions opposing those of AngII.[39] Collectively, these data show the complexity of this system, and illustrate why studies measuring singular markers of RAS may not reflect *in vivo* RAS activation.

In its updated view, the RAS is seen not only as an endocrine, but also a paracrine and an autocrine, system.[44] Importantly, accumulating evidence has shown that local RASs exist in various tissues, including the brain, heart, vasculature, and kidneys, and that each may be regulated independently of the circulating RAS.[39, 41, 42, 45] All of the components of the RAS – specifically, renin, AGT, and ACE – as well as their respective mRNAs, have been identified in renal tissue in a site-specific manner, allowing for intrarenal generation of AngII.[44, 46] Also, renal tissue concentrations of AngII are higher than can be explained by equilibration between plasma and the intrarenal extracellular fluid, further supporting the presence of an intrarenal RAS.[47]

Angiotensinogen is the only known precursor of the angiotensin peptides and its regulation may be of key importance to the activity of the intrarenal RAS.[48] While plasma AngII generation is mostly regulated by the release of renin into the circulation, in the kidney production of AngII is controlled by several independent mechanisms, of which intrarenal generation of AGT appears to be a critical factor.[49] An important distinction between the circulating and the intrarenal RAS is the fact that stimulation of renal AT_1R by AngII exerts a positive feedforward loop on this system particularly through, but likely not limited to, stimulation of production of AGT mRNA and protein.[42, 50] Proving this feedforward control, prorenin, renin, AGT, and ACE have all been shown to be upregulated within the kidneys of rodents undergoing long-term AngII infusion.[48] With a system that allows for continued augmentation of intrarenal generation and activity of RAS components, this local RAS appears to be of particular relevance in the pathophysiology of hypertension and progression of renal disease.[51, 52]

Effects on regulation of fibrosis and inflammation have been described for components of the RAAS other than AngII. Aldosterone, a mineralocorticoid produced primarily in the zona glomerulosa of the adrenal gland in response to increased extracellular potassium concentration and/or AT_1R stimulation by AngII,[53] is not only a key regulator of sodium, potassium and fluid balance, but also appears to contribute to the development of renal inflammation and fibrosis.[54] Its pro-inflammatory and pro-fibrotic effects are due, at least in part, to its influences on macrophage polarity, which favor the pro-inflammatory M1-macrophage phenotype, thereby increasing the production of pro-inflammatory cytokines such as TNF-α, chemokine (C-C motif) ligand 2 (CCL2) and CCL5, and promoting the release of TGF-β and plasminogen activator inhibitor-1 (PAI-1).[55] Additionally, prorenin, classically viewed as the inactive precursor form of renin, is able to interact with the (pro)renin receptor of several organs, including the heart and the kidney,

to induce a conformational change that allows it to become enzymatically active without undergoing proteolysis.[56] This change permits prorenin to not only influence regulation of local renin-angiotensin systems, but also to activate pro-inflammatory and pro-fibrotic signaling pathways, again including upregulation of TGF-β and PAI-1, as well as the extracellular matrix (ECM) components, fibronectin and collagen-1.[57]

In summary, by increasing the glomerular hydraulic pressure and the ultrafiltration of plasma proteins, and through the pro-inflammatory and pro-fibrotic actions of AngII and other components of this system, RAS activation contributes to both glomerular and tubulointerstitial injury and progression of chronic renal damage.[58, 59] The intrarenal RAS is now believed to play a critical role in the pathophysiology of CKD and hypertension.[42]

Proteinuria and the "overwork hypothesis"

Proteinuria, a well-documented marker of renal disease, is also hypothesized to promote renal damage directly.[60] In the healthy kidney, the glomerular filtration barrier prevents the loss of protein into the ultrafiltrate. This barrier is composed of fenestrated, glycocalyx-coated endothelium, a type IV collagen-rich glomerular basement membrane, laminin and heparan sulfate, and an epithelial layer comprised of podocytes; small gaps between the foot processes of the latter are bridged by small slit diaphragms that are critical to the selectivity of the filtration barrier.[61] The glomerular filtration barrier excludes molecules mostly based on their size, but also based on their charge.[62] As proteins are relatively large negatively-charged molecules, they tend to be retained within the lumen of glomerular capillaries. Small amounts of albumin and several smaller proteins are normally found in the ultrafiltrate, but these proteins undergo extensive degradation and/or tubular reabsorption, and are not detected by routine assays.[61] In addition to

the glomerular capillary tuft, the glomerulus contains mesangial cells that provide structure and support, produce the mesangial matrix, control capillary blood flow via cellular contraction, secrete growth factors, and perform housekeeping functions.[63] The glomerular and/or tubular dysfunction of certain disease states disrupt this well-organized process and may favor the development of renal proteinuria.

In the setting of CKD, single nephron adaptations, including glomerular hypertension, hyperfiltration, and hypertrophy, lead to mechanical damage of glomerular cells.[28] The podocyte layer is particularly susceptible to injury, as increased mechanical distention and shear stress at the filtration slits promote the detachment of podocytes from the glomerular basement membrane, ultimately worsening proteinuria and promoting further nephron loss.[31] Proteinuria is thought to cause direct mesangial cell injury with subsequent glomerulosclerosis, as well as tubulointerstitial injury and fibrosis.[64] In diseases associated with proteinuria, proximal tubular cells may be activated by increased levels of normal and novel urinary proteins that are thought to partially accumulate in the cytoplasm during the process of tubular reabsorption.[64] The activated proximal tubular cells may synthesize pro-inflammatory mediators, especially monocyte chemoattractant molecules (e.g., the chemokine regulated on activation, normal T cell expressed and secreted [RANTES], MCP-1, fractalkine, and complement component 3) and fibrosis-promoting molecules (e.g., endothelin, angiotensin II, and TGF-β).[65, 66] Both MCP-1 and RANTES, a nuclear factor-κB-dependent chemokine, have potent chemotactic activity for monocytes/macrophages and T lymphocytes, possibly mediating the tubulointerstitial injury that follows endocytosis of filtered proteins.[66] The inflammatory cascade triggered by proteinuria may damage the tubular basement membrane and facilitate the passage of tubular-derived products into the interstitium and peritubular capillaries, causing interstitial inflammation and eventual fibrosis.[65] In the distal

nephron, protein casts may also obstruct urinary flow, thereby worsening tubulointerstitial damage through barotrauma.[67]

In addition to evidence from basic science studies that supports the role of proteinuria in the aggravation of renal disease, clinical work in human beings has also consistently associated the level of proteinuria with the rate of progression of renal disease,[68] has shown the renoprotective effect of proteinuria reduction,[69, 70] and has identified proteinuria as an important modifiable risk factor for slowing the progression of chronic nephropathies.[71] As a result, reduction of the urinary protein-to-creatinine ratio (UPC) has become among the main goals for treatment of renal disease.[72]

Through its effects on glomerular hemodynamics, and pro-inflammatory and pro-fibrotic effects, persistent activation of the RAAS in animals with proteinuria may contribute to the progression of renal disease, worsening of proteinuria and development of mesangial cell proliferation and tubulointerstitial fibrosis.[59] Therefore, RAAS blockade is an integral part of the therapy of proteinuric CKD.[73]

Tubulointerstitial fibrosis and the "chronic hypoxia hypothesis"

While substantial scientific work appoints proteinuria as a critical mechanism for chronic kidney disease progression, in some renal diseases (e.g., hypertensive nephrosclerosis, a common cause of renal disease in human beings of Afro-Caribbean origin in the United States of America), tubulointerstitial injury progresses to end-stage renal disease in the absence of significant proteinuria.[74, 75] Conversely, some diseases characterized by heavy proteinuria (e.g., minimal change disease in children) lack significant interstitial fibrosis.[74] To address these inconsistencies, the "chronic hypoxia hypothesis" has emerged as an alternative unifying theory for renal disease

progression.[16] According to this theory, chronic oxygen deprivation of the tubulointerstitial compartment is the underlying cause of renal fibrosis. It is now believed that the progression of many forms of CKD is caused, at least in part, by a vicious cycle of hypoxia, renal inflammation, and fibrosis, which ultimately leads to renal failure.[76, 77]

Renal oxygenation is maintained within a relatively tight range through an intricate interplay between renal blood flow, glomerular filtration rate (GFR), oxygen consumption, and arteriovenous oxygen shunting.[78] Under a wide range of circumstances, renal autoregulation must balance the high renal oxygen demand imposed by tubular reabsorption, which is largely dependent on the active transmembrane transport of sodium via the Na-K-ATPase, with maintenance of adequate GFR. Furthermore, the renal vascular arrangement is such that vascular control is dominated by mechanisms that regulate GFR. Due to the complex interplay among these elements, the kidneys face unique challenges that make them particularly susceptible to hypoxia.[24, 78, 79] Factors that may contribute to renal hypoxia in CKD include increased oxygen and metabolic demand from hyperfiltration and tubular hyperfunction in uninjured nephrons; decreased capillary blood flow due to loss of peritubular capillaries; anemia; increased oxygen diffusion distance between peritubular capillaries and tubular and interstitial cells due to accumulating ECM; and an imbalance in vasoactive factors that favors vasoconstriction, predominantly via an increase in AngII and/or a decrease in vasodilatory factors (e.g., nitric oxide).[80-82] AngII-induced intrarenal vasoconstriction is evoked at the level of the efferent arterioles, with subsequent impact on perfusion of the postglomerular structures,[83] as well as the vasa recta, via pericyte contaction.[84] As renal oxygenation fails to meet tissue demands, a decrease in local oxygen tension triggers tubular injury, tubular cell apoptosis, activation of resident interstitial fibroblasts, myofibroblast differentiation, and recruitment of inflammatory cells, culminating in tubulointerstitial

inflammation and fibrosis, and further worsening local oxygen scarcity.[24, 32] Epithelial–to-mesenchymal transition (EMT) is also proposed to contribute to the pool of mesenchymal cells proliferating in the renal interstitium following hypoxia and subsequent tubular damage,[24, 32] although the contribution of this phenotypic change to the process of renal fibrosis remains controversial.[85-87] While early publications strongly implicate EMT in the development of fibrosis,[32] later studies, tracing genetic cell lineage, call into question the validity of these data.[88, 89] It appears that EMT's involvement in fibrosis may be relatively small when compared to that of other sources of myofibroblasts.[90]

At the molecular level, the cellular and tissular response to hypoxia is orchestrated by a milieu of interacting cytokines and transcription factors. The hypoxia-inducible factor (HIF) family controls the expression of hundreds of genes that regulate adaptive responses.[91] HIF-1 is a master regulator of oxygen homeostasis that plays critical roles in both cellular and systemic physiology and pathophysiology.[92] HIFs are heterodimeric transcription factors consisting of an oxygen-sensitive subunit (HIF-α) and a constitutively expressed subunit (HIF-β).[32, 75, 92] In normoxia, HIF-α proteins are continuously synthesized and degraded.[93] In hypoxia, HIF-α subunits are stabilized, dimerize with HIF-β, and bind to hypoxia-response elements in the regulatory regions of target genes.[32] Three different HIFα subunits – HIF-1α, HIF-2α and HIF-3α – have been described. However, research has focused largely on HIF-1α and HIF-2α.[92] In the hypoxic kidney, HIF-1α accumulates in tubular epithelial cells, whereas HIF-2α is found in glomerular cells, peritubular endothelial cells and fibroblasts.[94] Notable genes for which expression is regulated by HIF-1 include erythropoietin, endothelin-1, heme oxygenase-1, nitric oxide synthase, and vascular endothelial growth factor (VEGF).[92] While the role of HIFs in CKD remains to be fully elucidated, HIF-1α stabilization in hypoxic conditions has been implicated to

be a critical factor in the development of renal fibrosis and subsequent renal failure in mice.[95] Further, increased renal HIF-1α expression is associated with tubulointerstitial injury in the tissues from human patients with CKD.[96] It is proposed that activation of HIF-1 signaling may promote fibrogenesis by increasing expression of ECM–modifying factors and by facilitating EMT.[96]

The accumulation of ECM in response to hypoxia is thought to be caused by both increased matrix synthesis and decreased matrix degradation, due to decreased expression of matrix metalloproteinases (MMPs; matrix-degrading enzymes), and increased production of their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).[74] The MMPs are zinc-containing and zinc-dependent extracellular endopeptidases produced by several cell types, including macrophages, smooth muscle cells, endothelial cells, mesangial cells and fibroblasts.[97] With a broad range of substrates, MMPs are an important group of enzymes that regulate cell-matrix composition, playing a role in both normal and pathological processes, from embryogenesis and wound healing to cardiovascular disease and cancer.[98] At least 28 different MMPs have been described in human beings, and these are categorized according to their substrate specificity, sequence similarity and domain organization, as collagenases, gelatinases, stromelysins, matrilysins, membrane type, or other MMPs.[97]

The role of MMPs in the development of renal fibrosis is complex. In human renal fibroblasts, hypoxia has been shown to simultaneously stimulate ECM synthesis and suppress its turnover with increased production of collagen, decreased expression of the collagenase MMP-1, and increased expression of TIMP-1.[99] Despite their role in matrix degradation, MMPs are known to have both inhibitory and stimulatory roles in fibrosis.[100] Importantly, in addition to directly changing the architecture of the ECM, MMPs liberate bioactive fragments and growth factors, which influence cellular behavior.[101] In fact, studies in mice show that the actions of the gelatinases

MMP-2 and MMP-9,[77] and the matrilysin MMP-7, serve to worsen renal fibrosis.[102, 103] These pro-fibrotic effects are likely a consequence of proteolytic activation of latent growth factors and cell receptor–dependent activities.[102] The convoluted nature of MMPs' role in ECM metabolism is further illustrated by the fact that inhibition of MMP-2 and MMP-9 in mice with advanced stages of Alport syndrome accelerates renal fibrogenesis, whereas combined MMP inhibition before onset of tubulointerstitial fibrosis is protective.[104] Interestingly, the expression and activity of individual MMPs and TIMPs appears to be specific to particular forms of renal disease and distinct disease stages.[105] In adult human beings with CKD, serum MMP-2 activities were significantly increased and directly correlated with serum creatinine (sCr), while MMP-9 activities were significantly decreased and inversely correlated with sCr.[106] In children, on the other hand, serum concentrations of both MMP-2 and MMP-9, and their inhibitors TIMP-1 and TIMP-2, were elevated in CKD, and these concentrations were increased at the advanced kidney disease stages when compared to earlier ones.[107]

The relationship between MMP-7 and renal fibrosis has gained attention in current years. MMP-7 is now recognized as both a urinary biomarker of renal fibrosis in human beings, as well as an important mediator of fibrosis in CKD.[103, 108] Illustrating its value as a potential biomarker, MMP-7 expression was not detected in the kidneys of healthy humans and mice, but became measurable and increased as acute injury progressed to tubulointerstitial fibrosis in mouse models of renal disease.[109] In addition, in human patients undergoing cardiac surgery, urinary MMP-7 levels predicted the development of severe acute kidney injury (AKI) and poor in-hospital outcomes.[110] However, recent data from MMP-7 knockout mice subjected to renal ischemia-reperfusion suggest that this matrilysin may actually be protective in the setting of AKI by degrading FasL (a component of the extrinsic apoptotic pathway) and by mobilizing β-

catenin, thereby priming renal tubules for survival and regeneration.[111] Conversely, MMP-7 has been proposed to promote renal fibrosis via induction of EMT and ECM deposition, and through its close relationship with TGF-β signaling.[103] These data suggest that, as appears to be true for MMP-2 and MMP-9, the role of MMP-7 in renal disease is complex.

Transforming growth factor-β is a key player in tissue fibrosis and appears to play a major role in the pathogenesis of renal disease.[112] Mammalian TGF-β includes three major isoforms – TGF-β1, TGF-β2, and TGF-β3 – of which TGF-β1 is the predominant and best-characterized.[113] Both resident renal cells and infiltrating leukocytes may be stimulated to produce TGF-β in response to numerous factors, such as ischemia, AngII, endothelin-1, glucose, insulin, shear stress, insulin-like growth factor-1, atrial natriuretic factor, platelet activating factor, thromboxane, and TGF-β itself.[112] This growth factor exerts its biological functions mainly through the downstream signaling molecules, Smad2 and Smad3.[114] Activation of TGF-β triggers transcription of genes encoding matrix proteins, inhibitors of matrix-degrading enzymes and matrix-binding integrin receptors, as well as transformation of fibroblasts into myofibroblasts, and chemotaxis of fibroblasts and monocytes, thereby promoting the development of fibrosis.[112] In addition, TGF-β impairs renal autoregulation via generation of reactive oxygen species.[115]

Vascular endothelial growth factor, a proliferative, survival and trophic factor for endothelial cells, is also strongly stimulated by renal hypoxia.[116] The VEGF family is comprised of five members: VEGF-A (also known as VEGF), placental growth factor, VEGF-B, VEGF-C and VEGF-D. The first described member of the family, VEGF-A, is highly conserved between animal species.[117] This major factor for angiogenesis binds two tyrosine kinase receptors, VEGFR-1 and VEGFR-2, the latter exhibiting strong activity towards pro-angiogenic signals, and the former functioning as an endogenous VEGF inhibitor.[118]

Synergism between HIF-1 and the TGF- β1/Smad3 signaling pathway regulates VEGF gene expression at the transcriptional level.[119] It has been proposed that loss of peritubular capillary density, possibly modulated by decreased VEGF or other angiogenic factors, may contribute to progressive renal disease, as well as to the aging-associated decline in renal function.[120] Furthermore, in experimental models, VEGF was shown to preserve stressed endothelium, thereby stabilizing renal function and slowing histologic progression of renal disease.[116] Conversely, in a transgenic rabbit model, increased expression of glomerular VEGF resulted in glomerular hypertrophy and associated proteinuria.[121] Evidence also suggests that VEGF activation may be deleterious in the setting of early stage diabetic nephropathy, and that VEGF may play a role in adult polycystic kidney disease by increasing vascular permeability and promoting cyst formation.[116] While the role of VEGF in the pathogenesis of CKD remains ill-defined, clinical data suggest that plasma VEGF-A concentration is elevated in human beings with CKD.[122] In patients with diabetic nephropathy, urinary VEGF was also reported to be elevated, and positively associated with proteinuria.[123] Additionally, plasma VEGF-A levels have been found to be associated with progression to ESRD in patients with diabetic nephropathy.[124]

Finally, as discussed above, AngII, particularly intrarenal AngII, appears to be intricately linked to renal fibrosis. This important peptide contributes to renal hypoxia via both hemodynamic (e.g., constriction of efferent arterioles with subsequent hypoperfusion of postglomerular peritubular capillaries) and non-hemodynamic (e.g., induction of oxidative stress by activation of NADPH oxidase) mechanisms,[83] and also facilitates the development of tubulointerstitial fibrosis triggered by hypoxia.[40] Among other actions, AngII dramatically and synergistically potentiates TGF-β1–initiated α-smooth muscle actin expression and myofibroblastic transition in renal tubular cells.[125] In fact, TGF-β1 and reactive oxygen species appear to be the key mediators of the pro-

fibrotic effects of AngII, causing apoptosis and EMT of the renal tubular epithelium.[126] Additionally, AngII directly stimulates the expression of HIF-1α via the AT_2R in specific cell lines,[127] and infusion of AngII in mice causes renal HIF-1α activation and oxidative stress.[128] In rats subjected to 5/6 nephrectomy, treatment with the angiotensin receptor blocker (ARB) losartan alleviated renal fibrosis by down-regulating HIF-1α and upregulating MMP-9/TIMP-1.[129] In another study, losartan administered to rats prior to bilateral renal ischemia prevented the eventual development of CKD, even though it did not prevent or lessen the severity of the initial AKI.[130] Collectively, these data corroborate the link between RAS activation and the development of fibrosis, and support the renoprotective benefits of blockade of the former in CKD.

Canine chronic kidney disease – The role of proteinuria

As in other mammals, the self-perpetuating nature of CKD is well documented in dogs.[131] In fact, crucial scientific research, detailing single nephron adaptations to reduced nephron mass, has been performed in this species. Micropuncture studies using a canine surgical model of renal insufficiency documented glomerular capillary hypertrophy, hypertension and hyperfiltration, with associated increases in single nephron GFR that were proportional to the degree of renal ablation.[132, 133] Evidence from these and other studies using surgical models of canine CKD shows that a substantial reduction in functional renal mass is required for disease progression to occur following acute injury, and that when disease progression does occur, renal function declines in a fairly linear manner.[131] Interestingly, though proteinuria did develop in the canine remnant kidney model, and while its magnitude increased with advancing disease, UPC measured prior to the onset of disease progression was not predictive of the eventual rate of progression.[131] These data cast doubt on whether proteinuria is a mediator of renal injury in this model, suggesting that it may

instead represent a marker for that injury. However, due to the limited duration (4 months) of the follow-up period, longer-term deteriorations would have been missed. Since publication of this work, data from other experimental and clinical studies have supported the role of proteinuria in the progression of canine renal disease, although whether it represents merely a marker or also a mediator of disease remains to be definitively established.

Glomerular disease is a leading cause of canine CKD, affecting 52% to 90% of dogs with renal disease in early studies.[134, 135] In a large retrospective study of canine renal biopsy specimens obtained for suspected glomerular disease, immune-complex glomerulonephritis (ICGN) was diagnosed in nearly half (48.1%) of the cases.[136] In the same study, less common diagnoses included primary glomerulosclerosis, amyloidosis, nonimmune complex glomerulopathy, nonimmune complex nephropathy, and primary tubulointerstitial disease, recorded in 20.6%, 15.2%, 9.0%, 4.8% and 2.4% of cases, respectively.[136] As the description of canine glomerular disease continues to be refined, a standardized classification scheme has been proposed by the World Small Animal Veterinary Association Renal Pathology Initiative.[137] In collaboration with this group, the European Veterinary Renal Pathology Service recently reported a diagnosis of ICGN in approximately 50% of their cases,[138] consistent with the study referenced above. In this same study, non-ICGN (including minimal change disease, amyloidosis, and focal and segmental glomerulosclerosis pattern not associated with other glomerular diseases) was diagnosed in 36.4% of cases, and renal lesions not otherwise specified (including juvenile nephropathies and miscellaneous diseases) were identified in 13% of cases.[138]

Proteinuria is a hallmark feature of glomerular disease.[3] Although heavy proteinuria can be present irrespective of the specific diagnosis,[136] the magnitude of proteinuria, as assessed by the UPC, is significantly higher for cases of ICGN compared to most other glomerulopathies.[138,

[139] Nevertheless, significant overlap in UPC among categories precludes the use of the magnitude of proteinuria as a biomarker for glomerular disease type.

As in human beings, proteinuria has been linked to survival and disease progression in canine renal disease. In dogs with persistent proteinuria accompanying CKD, a UPC greater than 1.0 at initial diagnosis was associated with greater risk of development of uremic crises and death, and the relative risk of adverse outcomes increased by a factor of 1.5 for every 1.0-point increase in UPC.[140] A separate publication similarly found significantly shorter survival times in dogs with a UPC $\geq$ 1.0, as well as in those with systemic arterial hypertension (SAH).[141] In this study, both proteinuria and SAH were inversely correlated with GFR, as assessed by exogenous creatinine plasma clearance. Similarly, a recent study of factors associated with survival in dogs with CKD reported that dogs with overt proteinuria (i.e., UPC > 0.5) had an increased risk of death at any point during the study period, with a hazard ratio of approximately 3.2.[142] In contrast, Hokamp and colleagues found no association between UPC and survival time in dogs with naturally occurring CKD, although the use of antiproteinuric treatment, as well as the inclusion of several dogs with reversible glomerular injury, may have influenced these results.[139]

Further supporting the link between proteinuria and progression of renal disease, treatment with the angiotensin-converting enzyme inhibitor (ACEi) enalapril, at a dosage producing decreases in proteinuria magnitude, delayed the onset of azotemia progression in dogs with idiopathic glomerulonephritis.[143] Similar observations were made in Samoyed dogs with X-linked hereditary nephritis, for whom enalapril appeared to additionally provide a survival benefit, although the number of affected treated subjects (n = 5) was small.[144] Likewise, the ACEi benazepril has been shown to modulate progressive renal injury, presumably via reduction of glomerular and systemic hypertension and proteinuria, in dogs with induced renal insufficiency.[145]

As in human nephrology, RAAS blockade, historically achieved through ACE inhibition, is a critical part of antiproteinuric therapy in dogs.[146, 147] While ACEi have only modest antihypertensive effects, they appear to reduce proteinuria by a degree that exceeds what would be expected based on systemic BP reduction alone.[148] ACE inhibition reduces proteinuria by decreasing efferent glomerular arteriolar resistance, leading to decreased (normalized) glomerular transcapillary hydraulic pressure.[145] Other proposed beneficial effects of ACE inhibition include reduced loss of glomerular heparan sulfate, decreased glomerular capillary endothelial pore size, improved lipoprotein metabolism, slowed glomerular mesangial growth and proliferation, and inhibition of bradykinin degradation.[149] Although the antiproteinuric effects of both enalapril[143] and benazepril[150] have been documented in placebo-controlled canine studies, in a subset of dogs, proteinuria will not respond to, or will even worsen in the face of, ACE inhibition.[143] The reasons for poor response to ACEi in these dogs have not been dissected; however, ACE-independent generation of intrarenal AngII has been proposed as a major player in renal disease progression in human diabetic nephropathy,[151] and, if present in dogs, could explain, at least in part, the observed variability in the antiproteinuric efficacy of these drugs. While the intrarenal RAS has not been extensively studied in canine renal disease, intrarenal AngII immunoreactivity did correlate with sCr, glomerulosclerosis, cell infiltration and fibrosis in a small study of 20 dogs with CKD and three healthy dogs.[152] Interestingly, in a separate report, the same group of investigators found that ACE immunoreactivity was lower in kidneys of dogs with CKD than in normal kidneys, and quantitative analysis demonstrated a negative correlation between ACE and renal tissue damage in those same dogs.[153]

An alternative class of RAAS antagonist, the angiotensin II receptor blockers (ARBs), selectively block the AT_1R, the receptor type responsible for the potentially negative effects of

RAAS activation, which include systemic and renal vasoconstriction, increased renal sodium reabsorption, activation of inflammatory cytokines, vascular smooth muscle growth, oxidative stress, endothelial dysfunction, and increased PAI-1 activity and thrombosis.[37] Because ARBs directly block the action of AngII on the AT_1R, theoretically, these medications are able to antagonize AngII's effects whether this peptide was formed via ACE-dependent or -independent (e.g., via chymase) pathways.[154] This becomes increasingly relevant when considering the regulation of the intrarenal RAS, as chymase – a highly efficient AngII-generating enzyme[155] – appears to be an important producer of tissular AngII in the kidney.[156, 157] One additional theoretical benefit is that AT_1R blockade, by reduction of negative feedback inhibition, results in increased availability of AngII for stimulation of the unblocked AT_2R.[158]

Angiotensin receptor blockers are now widely used for the treatment of human renal and cardiovascular diseases.[159] A recent meta-analysis reviewed 20 randomized controlled trials that collectively studied over 25,000 people and compared the efficacy of the ARB telmisartan to those of placebo, an ACEi, other ARBs, non-RAAS-blocking antihypertensive drug therapy, or no medication.[160] Weighted evidence from this review favored telmisartan's efficacy for the improvement or prevention of proteinuria or albuminuria progression, both overall and within study categories (i.e., ARB-controlled, ACEi-controlled, and non-ARB/non-ACEi-controlled trials).[160] Conversely, a large network meta-analysis that included 119 randomized controlled trials evaluating a cumulative total of 64,768 people with CKD treated with different ACEi and ARBs concluded that ACEi were superior to ARBs and other BP-lowering agents at reducing the risk for renal failure, cardiovascular events, and death in people with CKD.[161] The question of which RAAS blocker class achieves better outcomes in renal disease is, to-date, a subject of debate. While no clinical studies have directly compared the efficacy of ACEi and ARBs for the treatment of

canine proteinuric renal disease, experimental work presented in abstract form compared the attenuation of the pressor response to exogenous AngI by the ACE inhibitor enalapril and the ARBs losartan and telmisartan in normal dogs.[162] Data from this study showed that telmisartan more completely blocked the pressor response to AngI than all other medications, and that losartan performed no or only minimally better than placebo. Additionally, one case report described clinical resolution of proteinuria in a dog treated with telmisartan following failure of complete response to ACEi treatment.[163]

In normal dogs, renal autoregulation limits changes in glomerular capillary pressure and GFR across a wide range of arterial BP by adjusting the vascular tone of the preglomerular arterioles; however, renal autoregulatory mechanisms are blunted in dogs with reduced renal mass.[164] Accordingly, in dogs with induced renal insufficiency and systemic arterial hypertension, magnitude of SAH is associated with renal injury, the latter characterized by decreased GFR, elevated UPC, and worse histologic renal lesion scores for mesangial matrix, tubule damage, and fibrosis.[165] Likewise, SAH is associated with albuminuria in dogs with naturally occurring CKD.[166]

Chronic kidney disease is a common cause of SAH. While the true prevalence of hypertension in canine CKD is difficult to determine, SAH has been documented in 9 to 93% of dogs with CKD,[167] and in 18 to 56% of dogs with glomerular disease.[168] These wide ranges likely reflect differences in measurement methodology, BP cut-offs used for the diagnosis of hypertension, and populations examined, as well as improved recognition of situational hypertension (i.e., "white-coat effect") in more recent studies.[169]

Because of the association between hypertension and worse renal outcomes[141] and the impact of systemic blood pressure on the regulation of intraglomerular pressure, initiation of additional measures for BP control is recommended for proteinuric dogs in which RAAS blockade

does not sufficiently reduce systolic BP.[146, 148] Calcium-channel blockers (CCBs) are typically used in such cases. Unlike RAAS blockers, CCBs predominantly vasodilate the afferent glomerular arteriole and their use may aggravate glomerular hypertension.[170, 171] Additionally, administration of the CCB amlodipine stimulated RAAS, as assessed by measurement of the urinary aldosterone-to-creatinine ratio (UACR), in dogs.[172] This stimulatory effect was blunted by co-administration of the ACEi enalapril. Further, in a study of hypertension in dogs with CKD, administration of amlodipine did not mitigate the risk of progression of CKD.[173] Therefore, the use of a CCB as a sole antihypertensive agent is not recommended in dogs with CKD.[148] A recent case series described the use of the ARB telmisartan as an adjunctive medication for treatment of five dogs with SAH refractory to the combination of benazepril and amlodipine.[174] In these dogs, two of which had a diagnosis of CKD, the combined administration of telmisartan and amlodipine resulted in successful and stable clinical control of systolic blood pressure (SBP).

In addition to RAAS blockade and, if needed, other antihypertensive measures, standard treatment of canine proteinuria includes dietary modification, with an emphasis on protein restriction and supplementation with polyunsaturated fatty acids (PUFAs).[146, 148] Evidence supporting dietary protein restriction and fatty acid supplementation originates from work performed in both CKD models and in naturally occurring disease. In dogs with X-linked hereditary nephropathy, consumption of a low-protein diet diminished proteinuria,[175] and feeding of a protein- and phosphorous-restricted diet reduced the severity of glomerular lesions and delayed progression to renal failure.[176] Likewise, data obtained from dogs with glomerular disease treated with benazepril suggest that a clinical “renal diet” may help to control proteinuria and blood pressure, without inducing clinically detectable malnutrition.[177]

The renoprotective effects of PUFA supplementation have been documented in dogs with surgically-induced renal insufficiency. Data from these canine models show that PUFAs reduce proteinuria, intraglomerular pressure and glomerular lesions, and maintain glomerular filtration rate.[178, 179] PUFA supplementation is also recommended in human beings with CKD, for whom proposed benefits include effects on renal function, albuminuria, lipoproteins, nutritional status, inflammation, thrombosis and clinical outcomes.[180]

In summary, current evidence suggests that antiproteinuric treatment is of benefit in canine CKD.[143, 181] As ARBs may be able to more completely block the renin-angiotensin system than ACEi,[162] they may be effective antiproteinuric agents in patients who fail standard therapy or may be candidates to replace ACEi as first-line therapy. Randomized controlled clinical trials comparing the antiproteinuric effects of ARBs with those of ACEi or placebo are presently lacking in the dog. Such studies in dogs receiving standard therapy, including a modified (protein-restricted) diet and PUFA supplementation, are needed before evidence-based therapeutic recommendations can be made.

Feline chronic kidney disease – The role of hypoxia and the renin-angiotensin-aldosterone system

Chronic kidney disease is the most common metabolic disease of domesticated cats.[182] Unlike dogs, primary glomerular disease is uncommon in this species.[182, 183] While several primary renal diseases have been identified in cats (e.g., amyloidosis, polycystic kidney disease, lymphoma, pyelonephritis, and AKI), an etiologic diagnosis is often not apparent in feline patients with CKD.[182, 183] Regardless of the inciting cause, the most common histopathologic renal lesion

in feline CKD is tubulointerstitial inflammation and fibrosis,[183] and as in human patients,[32, 184] this finding is strongly correlated to functional impairment.[185]

While its etiology is frequently unknown, several risk factors for feline CKD have been described. Interestingly, and in contrast to human beings, a diagnosis of diabetes mellitus or hepatic lipidosis is associated with decreased risk for CKD in domestic cats.[186] Risk factors for feline CKD include advanced age, thin body condition, prior periodontal disease or cystitis, being a neutered male (vs. a spayed female), and having undergone anesthesia or experienced documented dehydration in the year preceding diagnosis.[186, 187]

The fact that anesthesia and dehydration – both representing an opportunity for the development of transient systemic hypotension and decreased renal perfusion – are risk factors for CKD suggests that AKI, caused by transient reduction in renal perfusion or oxygenation, may be the inciting event for some cats with CKD. While many affected patients recover completely, it is well-established that AKI may lead to persistent renal disease; additionally, CKD is a risk factor for development of AKI.[188, 189] A growing body of scientific work indicates that AKI and CKD are not separate entities in people, but rather components of a closely interconnected disease continuum.[188, 190] Renal recovery from AKI is dependent on the balance between adaptive and maladaptive repair. Intertwined molecular pathways, such as inflammatory cascades and TGF-β signaling, are involved in both repair/regeneration and maladaptive responses.[189] The concept of an AKI-to-CKD continuum has also been considered in recent veterinary literature, with progressive CKD being described as a sequelae to recurrent or sustained renal injury.[191, 192] In both the veterinary and human medical fields, identification of biomarkers of active renal injury (e.g., neutrophil gelatinase–associated lipocalin[193-195]) is an active area of focused research, with the aim of ultimately using these biomarkers to differentiate AKI from progressive CKD. However, some

degree of overlap in biomarker levels between AKI and CKD cases is often observed, further strengthening the theory of interconnection between these syndromes.[191, 196]

Hypoxia and proximal tubular injury appear to play an important role in the AKI-to-CKD continuum.[197] As discussed in the sections above, accumulating evidence substantiates the role of chronic hypoxia as a central mechanism of development of renal fibrosis and progression of CKD.[198] Indeed, hypoxia, along with aging, appears to be among the best corroborated progression factors for feline CKD.[182] Schmiedt and colleagues recently reported a model of feline CKD that utilizes transient, unilateral, *in vivo* renal ischemia to induce fibrosis, interstitial inflammation, and tubular atrophy, changes that are also present in cats with naturally occurring CKD.[199, 200] These studies provide evidence that acute ischemic tubular injury in cats results in persistent changes that mimic those of CKD, and not only supports the link between AKI and CKD, but also corroborates the hypothesis that renal hypoxia may be an important player in the intrinsic progression of feline CKD. Additionally, this model affords a unique opportunity for studying the molecular pathways of progression of renal disease in this species.

Numerous down-stream mediators play an important role in the development of hypoxia-induced renal fibrosis. One of the most prominent, transforming growth factor-β1,[201] has been implicated in feline CKD. In cultured feline renal epithelial cells, incubation with TGF-β1 induces changes in cell morphology and alteration in gene expression consistent with the occurrence of EMT.[202, 203] This cytokine has also been evaluated in studies of client-owned cats with CKD. In an earlier study, TGF-β1 levels in urine (expressed as urinary TGF-β1-to-creatinine ratio), but not serum, were increased in cats with naturally occurring CKD (n = 23) as compared to healthy control cats (n = 13), suggesting that urinary TGF-β1 may be a valuable clinical marker of renal fibrosis.[204] A similar observation was made by the authors of a separate study, who also

documented significantly greater mean urinary TGF-β1-to-creatinine ratio in cats with CKD (n = 26) as compared to healthy cats (n = 18); a positive correlation between this ratio and sCr was identified, as well.[205] Urinary interleukin-8, MCP-1 and VEGF were also measured and normalized to urinary creatinine in this study.[205] As for TGF-β1, urinary interleukin-8 was significantly increased in CKD cats. While there was no difference in the MCP-1 levels between groups, cats with CKD had significantly lower urinary VEGF levels than did normal cats. This finding supports the previously mentioned hypothesis that decreased VEGF may be a contributing factor to loss of peritubular capillary density, promoting progressive renal disease.[120] Interestingly, in a study presented in abstract form, high urinary VEGF was associated with shorter survival and progression of azotemia in cats.[206] Therefore, as in human nephrology, the role of VEGF in feline CKD appears to be complex.

Although other mediators undoubtedly contribute, AngII promotes tubulointerstitial hypoxia by decreasing peritubular capillary perfusion (via preferential efferent glomerular arteriolar constriction), by creating a diffusion barrier to oxygen through direct stimulation of renal fibrosis, and by inducing oxidative stress.[83] Inappropriate and deleterious RAAS activation is well-documented in CKD in other species, and is a known factor for disease progression.[37] Research in cats has yielded inconsistent results, and the role of activation of the RAS in feline CKD has been called into question. However, RAAS has been shown to be inappropriately activated in some, but not all, models of feline CKD[207, 208] and in a subset of cats with naturally occurring disease.[209, 210] In a study comparing two techniques of inducing combined renal insufficiency and hypertension, serum aldosterone and PRA were significantly increased and positively correlated with SBP in cats undergoing a renal reduction-wrap, but not a renal reduction-alone, model.[207] In a later study examining the effects of benazepril on renal hypertension and chronic renal failure,

compared to baseline, SBP, PRA, and concentrations of circulating AngI, AngII and aldosterone were all elevated after 7/8 renal ablation.[208]

Few publications have assessed circulating RAAS status in naturally-occurring renal disease. In a study of hypertension associated with feline CKD, cats with CKD (n = 7) had higher mean PRA, and plasma AngI, AngII and aldosterone concentrations than did healthy control cats (n = 11).[210] Yet, in another study, in cats with untreated, hypertensive CKD (n = 12), mean PRA and plasma AngI concentrations were similar to those of healthy, normotensive cats (n = 5).[211] Although the mean values from the latter report appear to discredit the possible contribution of RAAS to feline hypertension in the context of CKD, circulating aldosterone was significantly higher in the CKD group. Further, PRA was either elevated or "normal" in the face of hypertension in five cats with markedly increased aldosterone, an inappropriate response that is consistent with RAAS activation. In a separate study of hypertensive CKD cats (n = 16), mean PRA was lower, while mean plasma aldosterone concentration was significantly higher, in diseased cats compared to healthy controls (n = 10).[212] Interestingly, PRA was higher than the mean value of controls (2.82 ng AngI/mL/hr) in three individual hypertensive CKD cats, with PRA being equal to or greater than 6.25 ng AngI/mL/hr in two of these cats. When PRA and plasma aldosterone concentrations were measured in normotensive and hypertensive cats with variable renal function (n = 196), mean PRA was lower in both azotemic and non-azotemic hypertensive cats, when compared to non-azotemic normotensive cats.[209] Azotemic hypertensive cats had a significantly higher plasma aldosterone concentration and aldosterone-to-renin ratio, suggesting that in these cats, plasma aldosterone elevation is independent of PRA.

The results of two recently published large, multicenter, placebo-controlled, randomized, double-masked clinical trials that evaluated the antihypertensive effects of the ARB telmisartan in

cats, support to the role of the RAAS in feline hypertension and CKD. [213, 214] Data from over 140 cats receiving telmisartan were collected in each study, 30[213] to 58%[214] of which were affected by CKD. Compared to placebo, telmisartan led to statistically and clinically significant BP reduction in both studies, confirming the contributory role of the RAAS in feline hypertension.

The presence and degree of RAAS activation appears to be extremely variable in feline CKD and hypertension; however, to-date, most investigations have evaluated components of the circulating, systemic RAAS, leaving the importance of the tissular system largely undescribed. As discussed above, recent focus on the role of the RAAS in the pathophysiology of organ injury has been extended to that of the local, tissular RAS. Notably, dissociation between intrarenal and plasma AngII levels has been shown in several animal models. For example, in rodent studies utilizing the remnant kidney model, activation of local renal tissue RAS is thought to contribute to progressive renal injury, despite the relatively low plasma renin levels seen in this model.[215] While little work has been done to characterize intrarenal RAS in companion animal species, one small veterinary study reported varying degrees of renin expression by the juxtaglomerular apparatus in the kidneys of cats with CKD (n = 10), with only 30% of these exhibiting increased expression compared to normal controls (n = 3).[216] In a more recent report, renal tubular and interstitial AngII immunoreactivity correlated with glomerulosclerosis and cell infiltration, but not tubulointerstitial fibrosis, in cats with naturally occurring CKD.[152] In these kidneys, there was no correlation between renin signal and plasma creatinine concentration, or between renin signal and any reported histopathological parameter. Similarly, a separate publication by this same group describing renal tissular ACE and ACE2 immunoreactivities in the same cats failed to identify correlation with examined histomorphological parameters.[153] These studies were limited by the number of tissue samples evaluated, both from affected (n = 11) and normal control (n = 2) animals, and by the fact

that immunohistochemistry, a relatively subjective method that allows only semi-quantification, was used to evaluate intrarenal RAS component expression.

The influence of RAS on feline glomerular hemodynamics has also been studied. As in dogs, micropuncture studies confirm the development of characteristic single nephron adaptations in the feline remnant kidney model.[181, 217] In this model, increases in single nephron glomerular filtration rate, associated with glomerular capillary hypertrophy and hyperfiltration, mesangial matrix expansion, and proteinuria, develop after partial renal ablation.[217] Underscoring the role of RAS in the glomerular hypertension of this model, treatment with benazepril effectively reduces glomerular capillary pressure and the ratio of efferent to afferent arteriolar vascular resistance, while augmenting single nephron GFR. The magnitude of glomerular capillary BP reduction seen with benazepril therapy is greater than what can be explained by the mild attendant decrease in systemic BP.[181] In the same studies, an antiproteinuric effect of benazepril was not apparent; however, proteinuria itself was mild in this model.

As primary glomerular disease is uncommon in cats, severe proteinuria is an infrequent feature of CKD in this species.[182] Nevertheless, of 251 cats diagnosed with CKD in a recently reported study, 15.4% were overtly proteinuric (i.e., UPC > 0.4) and 15.9% were borderline proteinuric (i.e., UPC, 0.2-0.4).[218] Importantly, feline proteinuria has been shown to be of prognostic significance, even if mild. In a study of 136 client-owned cats, 98 of which were azotemic, proteinuria was highly related to survival, even when accounting for severity of azotemia and age.[219] In this study, the hazard ratio for death or euthanasia was 2.9 for borderline proteinuric, and 4.0 for overtly proteinuric, cats. In a separate study of hypertensive cats (n = 141), the majority of which had evidence of renal disease, proteinuria before and after antihypertensive treatment with amlodipine was strongly associated with survival.[220] In yet another publication, the UPC of

cats that died within one month of diagnosis of CKD was significantly greater than the UPC of survivor cats.[221] Finally, the Benazepril in Renal Insufficiency in Cats (BENRIC) study also showed an inverse relationship between proteinuria and survival in cats with CKD.[222]

The role of the RAS in feline renal disease has been questioned. In the BENRIC study, treatment with benazepril decreased proteinuria severity, but did not significantly impact survival in cats with naturally occurring CKD. Experimental studies in healthy cats, evaluating the ability of various RAAS blockers to attenuate the pressor response to AngI, suggest that telmisartan achieves more complete and longer-lasting RAAS blockade than does benazepril,[223] suggesting that the apparent inability of ACEi to prolong survival may be due, as least in part, to incomplete RAAS inhibition by these drugs. Recently, a multicenter randomized, controlled, blinded clinical trial compared the efficacy of telmisartan and benazepril for the reduction of proteinuria in cats with CKD.[224] Telmisartan proved to be noninferior to benazepril and significantly decreased proteinuria relative to baseline at all assessment points, whereas benazepril did not. Whether telmisartan could provide a survival benefit in feline renal disease in not yet known.

In brief, accumulating evidence suggests a crucial role for hypoxia in the pathogenesis of tubulointerstitial renal fibrosis, from even the early stages of kidney disease. The development of an ischemic model of feline CKD[200, 225] offers an opportunity to study the effects of hypoxic insult on the feline kidney. If relevant molecular cascades are identified, therapeutic approaches that target this final common pathway should be effective against a broad range of renal diseases. Specifically, as supported by work performed in other species, MMPs,[226] TGF-β[227] and HIF[198] may prove to be potential targets for therapeutic intervention in feline CKD. Additionally, the role of RAS in feline CKD is poorly described and a source of debate. The use of renal disease models would allow concurrent characterization of both intrarenal and circulating RAS under conditions

for which the inciting insult is known. Should the data from such studies show a discrepancy between these two RASs, data from earlier studies assessing the circulating, systemic RAAS may need reinterpretation. Further, as data generated in renal disease models would be model-specific, similar evaluation of naturally occurring disease would be necessary to draw conclusions regarding mechanisms.

CHAPTER 3

EFFICACY OF TELMISARTAN FOR THE TREATMENT OF PERSISTENT CANINE RENAL PROTEINURIA[1]

[1] Bianca N. Lourenço, Amanda E. Coleman, Scott A. Brown, Chad W. Schmiedt, Roy D. Berghaus, Max C. Parkanzky, and Kate E. Creevy. To be submitted to the *Journal of Veterinary Internal Medicine*.

Abstract

Background**:** Information regarding the efficacy of the angiotensin II receptor blocker telmisartan for the treatment of proteinuria in dogs is limited.

Objective**:** To evaluate the safety and antiproteinuric efficacy of orally administered telmisartan in dogs with persistent, pathologic, renal proteinuria, when compared to enalapril.

Animals**:** Thirty-nine client-owned dogs with chronic kidney disease and urinary protein-to-creatinine ratio (UPC) ≥1.0 (if non-azotemic) or ≥0.5 (if azotemic).

Methods**:** In this prospective, randomized, double-masked clinical trial, dogs were block-randomized, according to presence or absence of azotemia and systemic arterial hypertension, to receive telmisartan (1.0 mg/kg PO) in the morning and an equal volume of placebo once daily in the evening, or enalapril (0.5 mg/kg PO) twice daily for 30 days. Thereafter, study drug dosages were increased every 30 days as necessary to achieve UPC ≤ 0.5. Dogs with UPC > 0.5 at day 90 were treated with the combination of telmisartan and enalapril. Percentage decrease in UPC at all timepoints compared to baseline was calculated and compared between treatment groups.

Results**:** A total of 39 (20 telmisartan-treated, 19 enalapril-treated) dogs were included. Median (range) baseline systolic blood pressure, blood creatinine concentration, and UPC were 154 mmHg (120-220), 0.9 mg/dL (0.7-5.0), and 4.7 (0.9-13.4), and 154 mmHg (126-210), 0.9 mg/dL (0.5-5.5), and 2.3 (0.8-15.5) for telmisartan- and enalapril-treated dogs, respectively. At day 30, percentage decrease in UPC was significantly greater for telmisartan-treated (-65.3% [-94.9%-104%]) versus enalapril-treated (-34.7% [-74.4%-86.5%]) dogs ($P = 0.002$). A greater proportion of telmisartan-, versus enalapril-treated dogs experienced ≥ 50% reduction in UPC at day 30, (16/20 [80.0%] and 5/17 [29.4%], respectively; $P = 0.003$). Compared to enalapril, telmisartan

treatment was associated with significantly greater reduction in UPC at days 60 and 90. No difference in change in UPC between groups was observed at day 120, when drug combination was allowed.

Conclusion and clinical significance**:** As a monotherapy, treatment with telmisartan resulted in a significantly greater decrease in UPC in proteinuric dogs, when compared to enalapril.

Abbreviations

ACE, Angiotensin converting enzyme

ACEi, Angiotensin-converting enzyme inhibitor

AngI, Angiotensin I

AngII, Angiotensin II

ARB, Angiotensin receptor blocker

AT_1R, Angiotensin II subtype 1 receptor

CKD, Chronic kidney disease

Cr, blood creatinine concentration

Hct, Hematocrit

IRIS, International Renal Interest Society

K, blood potassium concentration

PUFA, Polyunsaturated fatty acid

RAAS, Renin-angiotensin-aldosterone system

SAH, Systemic arterial hypertension

SBP, Systolic arterial blood pressure

UPC, Urinary protein-to-creatinine ratio

Introduction

Chronic kidney disease (CKD) affects up to 1.4% of the general canine population[10] and 10% of geriatric dogs presented to referral hospitals.[9] Of these, approximately 52 to 90% are affected by glomerular lesions,[134, 135] a hallmark of which is proteinuria.[3] In dogs, as in other species,[219, 222] proteinuria is a risk factor for disease progression, renal morbidity and mortality, and all-cause mortality.[140-142] Individuals with a urinary protein-to-creatinine ratio (UPC) > 1.0 are approximately 3 times more likely to experience uremic crisis and death than those with UPC ≤ 1.0.[140] As interventions that reduce the magnitude of proteinuria in affected individuals are associated with improved outcomes,[144, 176, 179] anti-proteinuric therapy is considered standard-of-care for dogs with proteinuric CKD.[146, 148]

Angiotensin-converting enzyme inhibitors (ACEi), such as enalapril, have been shown to decrease proteinuria in experimentally-induced[145] and naturally occurring canine CKD.[143, 144] By decreasing angiotensin II (AngII) production, ACEi's hemodynamic effects occur primarily via reduction of efferent glomerular arteriolar resistance, which lowers glomerular transcapillary hydraulic pressure, thereby reducing the magnitude of proteinuria.[228] Despite their overall benefit in lowering proteinuria within populations, ACEi are not universally successful, and a subset of dogs experience an increase in proteinuria despite therapy with enalapril.[143]

In human patients, angiotensin receptor blockers (ARBs) are commonly prescribed for the treatment of renal proteinuria.[229] The results of several clinical trials have shown that these drugs reliably reduce urinary protein loss and mitigate the progression from microalbuminuria to overt nephropathy.[230-232] The ARBs exert their effects on the AngII subtype 1 receptor (AT_1R), which mediates the adverse effects of AngII on the cardiovascular system and kidneys.[37] Selectivity for this receptor subtype grants drugs of the ARB class an advantage over ACEi, as the beneficial effects of AngII binding to AngII subtype 2 receptors are preserved.[158] Additionally, ARBs circumvent ACE-independent pathways, which are responsible for continued AngII production, particularly in the kidney, and would not be targeted by ACEi therapy.[158, 233]

While preclinical data in dogs suggests that the ARB, telmisartan, leads to superior angiotensin blockade when compared to enalapril,[162] information regarding efficacy of these drugs for treatment of canine proteinuria is limited.[163] The main objective of this study was to determine the short-term efficacy of telmisartan in reducing the magnitude of proteinuria in dogs with persistent pathologic renal proteinuria as compared to a standard oral dose of enalapril. The authors hypothesized that telmisartan would lead to a greater reduction in UPC than would enalapril after one month of therapy. This study's secondary objectives were to evaluate the relative abilities of telmisartan and enalapril, when administered at a progressively increasing dosages, to reduce proteinuria in the same population over a 2-month period, and to determine whether treatment with a combination of telmisartan and enalapril for 30 days would lead to a clinically significant reduction of UPC in dogs who are persistently proteinuric on 'ceiling doses' of either medication alone. We hypothesized that telmisartan would lead to a greater and faster reduction in UPC than would enalapril, and that clinically significant reductions in UPC would

be noted with combination therapy. A final objective of this study was to evaluate the safety of telmisartan when administered at progressively increasing dosages, and when co-administered with enalapril, in a population of dogs with naturally occurring CKD.

Materials and methods

Animals**.** In this prospective, randomized, double-masked clinical trial, proteinuric dogs were recruited prospectively from patients presented to the Veterinary Teaching Hospital of the University of Georgia. Azotemic and non-azotemic dogs with hypertensive and non-hypertensive CKD were included if they had confirmed persistent, pathologic, renal proteinuria due to CKD, based on fulfillment of the criteria described below.

Inclusion criteria. Dogs were considered for enrollment if they had persistent proteinuria with a UPC ≥ 1.0 (if non-azotemic, i.e., blood creatinine [Cr] < 1.4 mg/dL; IRIS CKD stage 1) or ≥ 0.5 (if azotemic, i.e., Cr ≥ 1.4 mg/dL; IRIS CKD stages 2-4), documented in ≥ 2 separate urine samples collected ≥ 14 days apart; abdominal ultrasound findings consistent with CKD, and absence of renal neoplasia or causes for post-renal proteinuria (e.g., urolithiasis, urinary tract neoplasia).

Exclusion criteria. Dogs were excluded if one or more of the following were identified: evidence of hemorrhage, inflammation or bacteria on urine sediment analysis; positive urine culture at the time of identification of proteinuria; positive heartworm antigen test within 3 months of identification of proteinuria and/or not currently receiving regular monthly heartworm preventive; historical, physical examination or clinical pathologic findings suggestive of acute kidney injury, infectious nephropathy or lower urinary tract disease; SBP < 120 mmHg;

moderate-to-severe hyperkalemia (blood potassium concentration [K] > 6.5 mmol/L); history of having received oral ACEi and/or corticosteroids in the 14 days preceding examination; concurrent illness associated with proteinuria, the treatment of which might result in mitigation of proteinuria (e.g., systemic lupus erythematosus, ehrlichiosis, neoplasia). Dogs with suspected or confirmed hyperadrenocorticism and diabetes mellitus were included if their disease was considered clinically and biochemically controlled with ongoing medical therapy.

Patient grouping for block-randomization. At enrollment, dogs were categorized according to presence or absence of systemic arterial hypertension (SAH) and azotemia. For the purposes of this study, SAH and azotemia were defined for dogs in which average indirect systolic arterial blood pressure (SBP) was ≥150 mmHg and Cr was ≥ 1.4 mg/dL, respectively. Based on this categorization, dogs were designated as belonging to one of four groups: non-azotemic, normotensive; non-azotemic, hypertensive; azotemic, normotensive; and azotemic, hypertensive. Within each group, dogs were block-randomized to treatment group in groups of 4. The investigators remained masked to each patient's treatment group for the duration of the study.

Screening/baseline evaluation. The following baseline data were collected for each case: indirect SBP measurement using Doppler sphygmomanometry, full physical examination (performed by one of the study investigators), serum biochemistry panel, blood gas panel, urinalysis, UPC, urine culture, abdominal ultrasonographic examination (performed by a board-certified veterinary radiologist or a trainee under the supervision of a radiologist), and either a single *Dirofilaria immitis* antigen test[a] or a combined *Anaplasma phagocytophilum*, *Anaplasma platys*, and *Borrelia burgdorferi* and *Dirofilaria immitis* antigen test.[b] Systolic blood pressure was measured following an acclimation period of at least 10 minutes and before physical

examination, in keeping with guidelines set forth by the American College of Veterinary Internal Medicine.[167] Briefly, a cuff with width corresponding to 30-40% of the circumference of the extremity at the site of cuff placement was chosen. The first measurement was discarded and the average of five consecutive consistent measurements was recorded. If there was substantial variation, the readings were discarded and the process repeated until consistent consecutive measurements were obtained. Laboratory analyses were performed by the Clinical Pathology Laboratory of the University of Georgia College of Veterinary Medicine.

Endocrine disease testing, including ACTH stimulation and/or low dose dexamethasone suppression testing and thyroid hormone measurement, was performed if clinical signs or results of baseline testing raised suspicion for hyperadrenocorticism or hypothyroidism.

The results of screening tests, if performed within 14 days (for laboratory data) or eight weeks (for diagnostic imaging) of inclusion in the study, could be used as baseline information, as long as there were no changes to the history or physical examination findings in the interim.

Whenever possible, baseline UPC was defined as the average of two measurements, obtained within 30 days of enrollment in the absence of an active urinary sediment, urinary tract infection, or treatment with RAAS blockers or corticosteroids. If an earlier UPC result was obtained > 30 days prior to enrollment, UPC measured at baseline (day 0) was used as the baseline value.

Prior to inclusion, owners were required to read and sign a form consenting to their pets' participation in the study.

Angiotensin receptor blocker/angiotensin-converting enzyme inhibitor therapy. Dogs were randomized to receive to receive oral telmisartan at a dosage of 1.0 mg/kg in the morning

and an equal volume of placebo once daily in the evening, or oral enalapril at a dosage of 0.5 mg/kg twice daily, in a double-masked manner. Concentrations of telmisartan solution[c] (10 mg/mL) and enalapril suspension[d] (5 mg/mL) allowed for equivalency of volume per kg body weight administered at each dose (0.1 mL/kg) regardless of treatment group.

Concurrent antihypertensive therapy. For dogs in which persistent, severe SAH (SBP ≥ 180 mmHg)[167] was documented, the calcium channel blocker amlodipine was started contemporaneously at a dosage of 0.1 mg/kg once daily. During the 120 days of the study, amlodipine dosage was increased or decreased at the clinician's discretion in order to maintain SBP of 100 to 180 mmHg. If at any point in the study a dog became hypotensive, amlodipine dosage was decreased at the discretion of the attending clinician and the dog's blood pressure was re-evaluated in 6-8 days. For the purposes of this study, hypotension was defined as SBP <100 mmHg in a dog with clinical signs of hypotension (e.g., weakness, lethargy and/or syncope).

Nutritional therapy**.** Unless contraindicated, dogs were maintained on a commercially available renal diet or homemade diet formulated by a certified veterinary nutritionist to be low in phosphorus and protein, alongside a polyunsaturated fatty acid (PUFA) supplement. Nutritional intervention (i.e., diet and PUFA supplement) was initiated at least 14 days prior to enrollment, and remained constant during the study period.

Monitoring. Following initiation of therapy with the study drug, dogs were followed for 120 days. The first 30 days of the study were intended to assess and compare the short-term antiproteinuric effects of telmisartan and enalapril (study phase I). The following 60 days (days 30 through 90; study phase II) were planned to assess the efficacy of these drugs when administered at progressively increasing dosages. The final 30 days of the study (days 90

through 120; study phase III) were designated to assess the efficacy of the combination of the two study drugs for patients whose proteinuria was not well controlled during the earlier phases.

The monitoring protocol in the present study followed the recommendations of the American College of Veterinary Internal Medicine for standard therapy of canine glomerular disease.[148]

Study phase I**.** An overview of study phase I is depicted in figure 3.1. All dogs were rechecked on day 7 ± 1, at which time physical examination, SBP, Cr and K were evaluated. An increase in Cr of > 30% compared to baseline or identification of moderate/severe hyperkalemia (i.e., K > 6.5 mmol/L) or systolic hypotension prompted investigator unmasking and removal of the patient from the study. For dogs who remained severely hypertensive (i.e., SBP ≥ 180 mmHg) amlodipine was up-titrated to 0.1 mg/kg PO BID. Thereafter, severely hypertensive dogs were rechecked at 7-day intervals. At each visit, if average SBP measurements remained ≥ 180 mmHg, the dosage of amlodipine was increased in increments of 0.05 mg/kg q12h to a maximum dose of 0.3 mg/kg q12h. Systolic blood pressure and Cr were rechecked 7 ± 1 days following any adjustments.

On day 30 ± 2, all dogs underwent physical examination, SBP, serum biochemistry, urinalysis and UPC measurement. To account for known day-to-day variability, UPC was determined by pooling three voided urine samples of equal volume at all post-enrollment time points, as previously described.[234] Owners were instructed to collect and refrigerate a mid-stream urine sample in a sterile urine collection cup on each of the three consecutive mornings preceding the recheck visit. All samples were analyzed within 3 days of collection. Patients for which pooled-sample UPC was ≤ 0.5 were maintained on the starting dosage of the study

medication and unless otherwise indicated, were not rechecked until the end of the study (day 120).

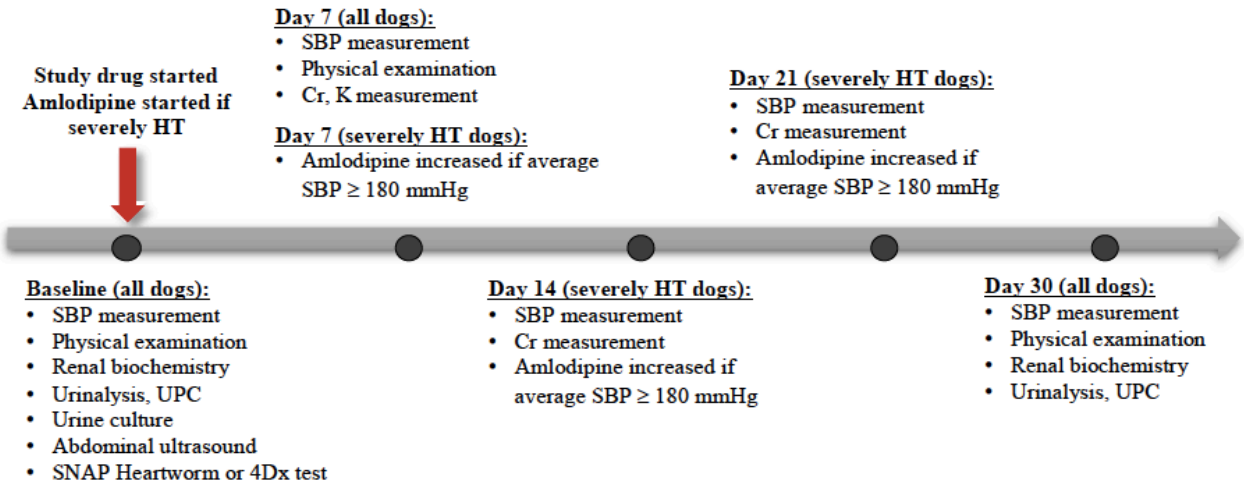

Figure 3.1. Overview of study design for phase I (short-term efficacy).

Abbreviations: HT, hypertensive; SBP, systolic blood pressure in mmHg; Cr, blood creatinine concentration; K, blood potassium; UPC, urinary protein-to-creatinine ratio.

Phase II visits**.** An overview of study phases II and III is depicted in figure 3.2. At day 30 ± 2, in patients for which UPC was > 0.5, up-titration of study medication was performed. For the telmisartan group, dosage was increased to 2 mg/kg PO in the morning and equivalent volume of placebo in the evening, and for the enalapril group, dosage was increased to 1.0 mg/kg PO BID. The response to up-titration was assessed at day 60 ± 2, at which point UPC was measured on a pooled sample. As for day 30, patients for which the UPC was ≤ 0.5 at day 60 had no medication adjustments and were not rechecked until day 120. For those dogs with UPC > 0.5 at day 60, dosage up-titration – to 3 mg/kg PO in the morning and equivalent volume of placebo

in the evening for telmisartan-treated dogs and to 1.5 mg/kg PO BID for enalapril-treated dogs – was performed. At day 90 ± 2 (final phase II visit), a similar assessment was performed, and in those patients with UPC > 0.5, the other study medication was added, at 1.0 mg/kg PO in the morning and an equal volume of placebo once daily in the evening for telmisartan (in enalapril-treated dogs), or 0.5 mg/kg PO BID for enalapril (in telmisartan-treated dogs).

Dogs were rechecked 7 ± 1 days following any study drug up-titration or addition, at which time physical examination, SBP, Cr and K were evaluated. At any recheck, an increase in Cr of > 30% compared to baseline, identification of moderate/severe hyperkalemia, or documentation of systolic hypotension prompted investigator unmasking and removal of the patient from the study.

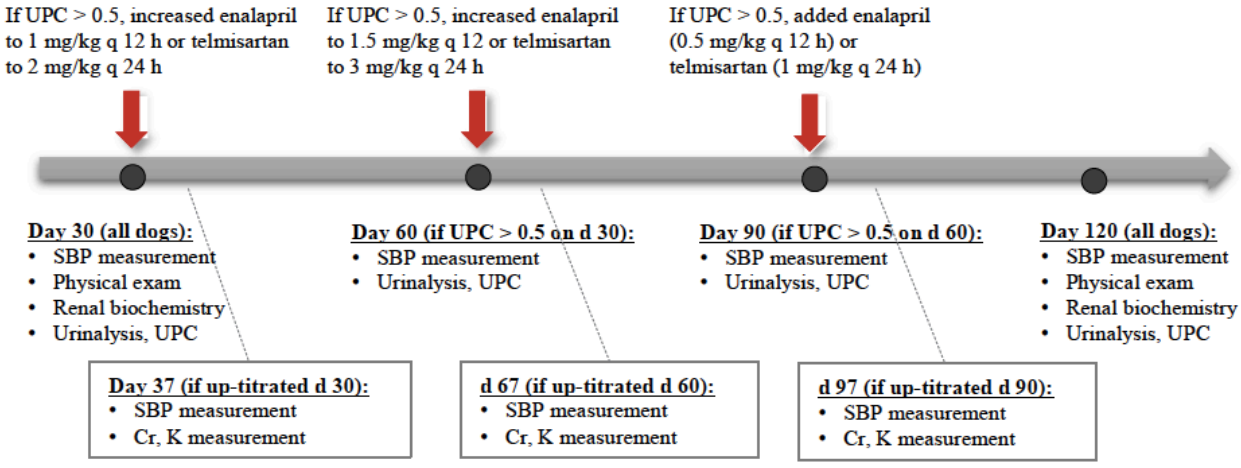

Figure 3.2. Overview of study design for phases II (intermediate-term efficacy) and III (efficacy of combination therapy).

Abbreviations: SBP, systolic blood pressure; Cr, blood creatinine concentration; K, blood potassium; UPC, urinary protein-to-creatinine ratio.

Phase III final study visit**.** All dogs were rechecked on day 120 ± 2, at which time SBP, Cr and K, urinalysis and UPC were evaluated.

Populations considered. Dogs that were randomized and received at least 1 dose of study medication comprised the intention-to-treat population. Dogs that successfully fulfilled all eligibility criteria, had analyzable data available from at least the first scheduled visit (day 30 ± 2) and that adhered to the study protocol with no major deviations, comprised the per-protocol population.

Primary outcome variables. The primary outcome variables with respect to efficacy were percentage change in UPC from baseline to day 30 ± 2 (phase I; short-term), baseline to days 60 ± 2 and 90 ± 2 (phase II; intermediate-term), and baseline to day 120 ± 2 (phase III; drug combination phase). Percentage change in UPC was calculated by subtracting baseline UPC from UPC at the time point of interest, and then diving the resulting difference by the baseline UPC.

Secondary outcome variables. Additional efficacy outcomes of interest included the proportion of dogs and odds of achieving ≥ 50% UPC reduction, and proportion of dogs achieving UPC ≤ 0.5 at each of days 30 ± 2, 60 ± 2, 90 ± 2, and 120 ± 2, as well as time to 50% UPC reduction and time to UPC ≤ 0.5, and the proportion of dogs for which the second medication was added.

Safety outcomes of interest included percentage change from baseline in Cr, K and hematocrit (Hct) at each of days 30 ± 2, 37 ± 1, 67 ± 1, 97 ± 1 and 120 ± 2, calculated in the same manner as for the percentage change in UPC. The proportion of dogs removed due to likely drug-related (i.e., azotemia and hyperkalemia) and unrelated (e.g., development of comorbid

condition such as pancreatitis or hepatopathy) adverse events at any time during the study period was also compared.

Adverse events. At each visit, owners were asked open-ended questions, in the form of a written questionnaire, regarding any observed changes in their pet since the preceding visit. An adverse event was defined as any unfavorable or unintended observation reported by the owner while the dog was enrolled in the study, regardless of whether it was believed related to study medication.

Sample size calculation**.** Because of considerable daily variation in UPC in dogs with stable proteinuria, serial UPC must differ by >40% to conclude with confidence that reduction is in response to therapy.[146] Therefore, percentage change in UPC of ≥50% was considered clinically significant for the purposes of this study. Mean UPC reduction of 51% is expected in a population of proteinuric animals treated with enalapril.[14] An initial sample size calculation, assuming a 5% alpha error level, demonstrated that 27 dogs per treatment group would provide adequate statistical power (80%) to identify a 50% change in UPC compared to baseline, utilizing a two-tailed test. Post-hoc sample size calculation, using data from the present study population and targeting detection of a 20% difference in percentage change in UPC between treatment groups, was performed. Assuming a 5% error level and a standard deviation in this outcome variable of 0.4 (40%), it was determined that 128 dogs (64 per treatment group) would provide 80% power to detect a 20% difference in percentage change in UPC between groups.

Interim monitoring**.** Interim analysis was scheduled for January 2019. The data obtained from the first 30 days of enrollment (phase I) for each dog was used to compare percentage change in UPC and proportion of dogs achieving a ≥ 50% UPC reduction between the two treatment groups. It was defined *a priori* that the trial would be terminated if there was a

statistically significant difference in percentage change in UPC between the two study groups at day 30, and the median percentage UPC reduction in at least one of treatment group was ≥ 50% of baseline (i.e., if the UPC reduction induced by at least one of the drugs was deemed clinically relevant) at day 30. A uniform alpha-spending function was used to determine significance level, with the number of dogs recruited by that time used as the information fraction (t*).[235]

Interim analysis was conducted by an independent statistician, who did not participate in recruitment, management or follow-up of study cases (RDB). All other investigators remained masked to individual drug assignments, as well as to the identity of a possible superior drug, until all enrolled dogs completed the study.

Statistical analyses**.** Statistical analyses were performed using commercially available software packages.[e,f] A significance level of 0.05 was used for all analyses. Data were examined for normality by visual assessment of histograms and normal quantile plot, and the Shapiro-Wilk test. Normally distributed data were presented as mean ± SD and compared between groups using the Student's t test. Non-normally distributed data were presented as median (range) and compared using the Wilcoxon rank sum test. Proportion of dogs achieving a given endpoint were compared using the Fisher's exact test. Time to secondary efficacy endpoints was compared using the Kaplan Meier estimator function and the logrank test.

Analyses were performed on the intention-to-treat population. Dogs not evaluated per study protocol or removed prior to a given visit were treated as having missing data. Due to the unintended inclusion and subsequent randomization of a dog developing an active infectious disease (*Borrelia burgdorferi*), select comparisons were also performed as a per protocol analysis, and are reported as such.

Results

Animals. A total of 48 dogs were screened for eligibility between January 1, 2015 and December 31, 2018. Of these, 39 dogs met the enrollment criteria and were included in the intention-to-treat population (Figure 3.3).

An interim analysis, performed using the first 30-day data collected from these 39 dogs, revealed a both statistically significant and clinically relevant difference between drugs, and enrollment was therefore terminated in January 2019. Study participation was ongoing for six dogs at the time of interim analysis. All had a documented reduction in UPC at their latest evaluation, with the last UPC measured being ≤ 0.5 for four of these dogs. Management of these 6 cases was completed in a double-masked manner.

Baseline demographic and clinical characteristics were similar between the two study groups (Table 3.1).

Historical or concurrent diseases were reported in all but five enalapril- and one telmisartan-treated dogs (Table 3.2). A summary of patient diet, fish oil supplementation and concurrent medications (other than ectoparasites and heartworm prevention) data for the study population are presented in table 3.3.

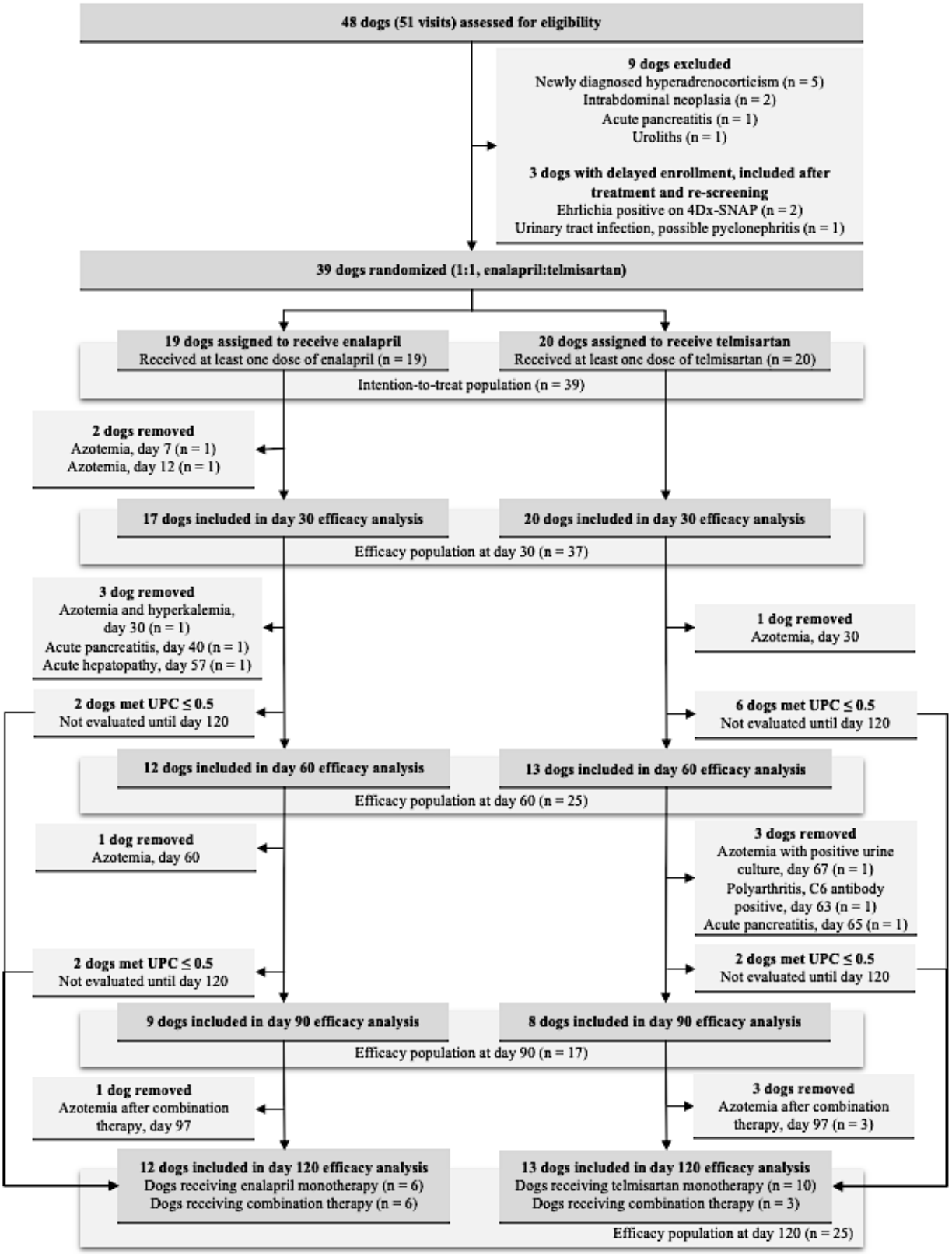

Figure 3.3. Flow diagram illustrating the progress of patients through the present clinical trial.

Table 3.1. Baseline demographic, clinical and laboratory data from telmisartan-treated (n = 20) and enalapril-treated (n = 19) dogs (intention-to-treat population). Data are presented as mean ± SD or median (range) where appropriate.

		Enalapril group	**Telmisartan group**
Number		19	20
Age (years)		10.2 ± 2.3 10.4 (3.1-14.5)	8.8 ± 2.8 8.75 (4.3-14.9)
Gender (n)			
	Female spayed	13	14
	Male neutered	6	6
Breed (n)			
	Jack Russell terrier	4	0
	Beagle	2	2
	Boston terrier	0	2
	Fox terrier	0	2
	Golden retriever	2	0
	Miniature Schnauzer	0	2
	Yorkshire terrier	2	0
	Mixed breed	1	2
	Other (frequency ≤ 1)	8	10
Body weight (kg)		13.4 (3.5-41.3)	11.5 (4-42.8)
Systolic blood pressure (mmHg)		154 (126-210)	154 (120-220)
Serum creatinine concentration (mg/dL)		0.9 (0.5-5.5)	0.9 (0.7-5.0)
Blood urea nitrogen concentration (mg/dL)*		11 (8-101)	13 (5-64)
Serum potassium concentration (mmol/L)		4.42 ± 0.47 4.32 (3.89-5.5)	4.38 ± 0.25 4.39 (3.88-4.88)
Serum albumin (g/dL)**		3.4 (1.7-3.9)	3.3 (2.2-3.9)
Hematocrit (%)		45 (35-53)	46 (29-53)
Urinary protein-to-creatinine ratio***		2.29 (0.91-15.54)	4.65 (0.90-13.39)
Study group (n)			
	A	6	6
	B	9	8
	C	1	2
	D	3	4
IRIS CKD stage (n)			
	1	15	14
	2	1	3
	3	2	3
	4	1	0

For ease of comparison, both mean and median are presented for variables with discordant assessment for approximation to normal distribution between groups.

* For one dog, blood urea nitrogen concentration was above the limit of reporting for the assay (> 100 mg/dL) and was here assigned a value of 101 mg/dL.

** Albumin was measured at day 0 for 14 telmisartan- and 17 enalapril-treated dogs.

*** Average of two measurements within the preceding 30 days of enrollment, when available.

Table 3.2. Summary of historical or concurrent conditions for telmisartan-treated (n = 20) and enalapril-treated (n = 19) dogs (intention-to-treat population). Data are presented as number of dogs (%).

		Enalapril group	**Telmisartan group**
	Number	19	20
Concurrent conditions	Orthopedic disease	5 (26.3%)	6 (30%)
	Periodontal disease	5 (26.3%)	5 (25%)
	ACVIM stage B1 MMVD	3 (15.8%)	2 (10%)
	Heart murmur with open diagnosis	2 (10.5%)	3 (15%)
	Hypothyroidism	2 (10.5%)	2 (10%)
	Atypical hyperadrenocorticism	0	1 (5%)
	Urinary incontinence	2 (10.5%)	2 (10%)
	Chronic pancreatitis	0	3 (15%)
	Food-responsive enteropathy	1 (5.3%)	0
	Stress colitis	0	1 (5%)
	Ocular disease	2 (10.5%)	3 (15%)
	Chronic respiratory disease	2 (10.5%)	1 (5%)
	Nodular hepatopathy	3 (15.8%)	1 (5%)
	Gall bladder mucocele	1 (5.3%)	0
	Atopic dermatitis	1 (5.3%)	2 (10%)
	Aural hematoma	1 (5.3%)	0
	Acral lick granuloma	0	1 (5%)
Relevant historical conditions	Completely excised neoplasia	5 (26.3%)	3 (15%)
	Hepatosplenic infarction	0	1 (5%)
	Acute pancreatitis	1 (5.3%)	0
	Intervertebral disc disease	1 (5.3%)	0
	Immune-mediated thrombocytopenia	0	1 (5%)
	None reported	5 (26.3%)	1 (5%)

Abbreviation: MMVD, myxomatous mitral valve disease.

Table 3.3. Summary of concurrent medications, nutritional therapy and polyunsaturated fatty acid supplementation data from telmisartan-treated (n = 20) and enalapril-treated (n = 19) dogs (intention-to-treat population). Data are presented as number of dogs (%).

		Enalapril group	**Telmisartan group**
	Number	19	20
Clinical renal diet	Yes	17 (89.5%)	15 (75%)
	No	2 (10.5%)	5 (25%)
Polyunsaturated fatty acid supplementation	Yes	18 (94.7%)	16 (80%)
	No	1 (5.3%)	4 (20%)
Concurrent oral medications	Levothyroxine	2 (10.5%)	2 (10%)
	Trilostane	0	1 (5%)
	Phenylpropanolamine	1 (5.3%)	2 (10%)
	Ursodeoxycholic acid	2 (10.5%)	1 (5%)
	S-Adenosylmethionine + silybin	0	2 (10)
	Pimobendan	1 (5.3%)	1 (5%)
	Amlodipine*	1 (5.3%)	1 (5%)
	Gabapentin	0	1 (5%)
	Tramadol	3 (15.8%)	0
	Trazodone	1 (5.3%)	1 (5%)
	Amantadine	0	1 (5%)
	Carprofen	0	1 (5%)
	Clopidogrel	0	1 (5%)
	Maropitant	0	1 (5%)
	Famotidine	1 (5.3%)	0
	Metoclopramide	0	1 (5%)
	Metronidazole	0	1 (5%)
	Theophylline	0	1 (5%)
	Hydrocodone	0	1 (5%)
	Diphenhydramine	0	1 (5%)
Ophthalmic ointments	Cyclosporine	1 (5.3%)	0
	Neomycin/Polymyxin B/Gramicidin	1 (5.3%)	0
	Neomycin/Polymyxin B	1 (5.3%)	0
Injectable	Canine Atopic Dermatitis Immune IL-31 monthly	0	1 (5%)

* Amlodipine is listed as concurrent medication here only if administration was initiated prior to study enrollment – total number of dogs receiving amlodipine is described in the text.

Note: Concurrent medications listed if administered at any point during study participation

Protocol adherence and completion of study period. Deviations from study protocol or documented lack of owner compliance occurred during the study period in four dogs. In three cases (two telmisartan- and one enalapril-treated), deviation from study protocol occurred when it was deemed by the study investigators that study drug up-titration or combination therapy to target a UPC ≤ 0.5 would pose undue risk to a patient experiencing one or both of the following: a plateau in change in UPC, or progressive azotemia that approached, but did not meet, the criteria for removal (i.e., 30% increase in Cr) in response to prior up-titrations. These deviations occurred at study day 60 in one dog (second up-titration not performed; dog removed following the development of severe, acute pancreatitis at day 65) and at study day 90 in two dogs (i.e., combination therapy not prescribed). In one telmisartan-treated dog, administration of study drugs was discontinued by the owner at day 93, in response to a period of lethargy and inappetence that developed after combination therapy was initiated at day 90. Evaluation of this dog's renal biochemistry panel at day 97 revealed progressive azotemia and prompted patient removal from the study, unmasking and temporary discontinuation of all RAAS blockers. As two of the four dogs for which a protocol deviation or known lack of compliance occurred were removed from the study prior to their next assigned UPC evaluation, the potential impact of such deviations was limited to data from day 120 for only two dogs, one dog in each treatment group.

A total of 25 (13 telmisartan-treated and 12 enalapril-treated) dogs completed the 120-day study period (Figure 3.3). Seven dogs in each treatment group were removed prior to day 120. Five dogs in each group were removed for reasons deemed likely related to administration of RAAS blockers. Progressive azotemia (percentage change in Cr > 30%) was noted in all 10 of

these dogs. Of these, three dogs in the telmisartan group and one dog in the enalapril group became azotemic following initiation of combination therapy at day 90 and were subsequently removed at day 97. Of those removed for azotemia while on monotherapy (i.e., during the first 90 days), one enalapril-treated dog, which was removed at day 30, also experienced concurrent hyperkalemia (K, 7.56 mmol/L) that would have prompted removal as well. One telmisartan-treated dog removed for progressive azotemia at day 67 displayed systemic signs of illness (i.e., vomiting and diarrhea) and had documented bacteriuria and a positive urine culture (*E. coli*, >100,000 cfu/mL) at the time of removal. Thus, concurrent pyelonephritis could not be ruled out.

Two dogs in each group were removed for reasons deemed likely unrelated to administration of study drugs. One dog in each group experienced an episode of severe, acute pancreatitis, and one enalapril-treated dog suffered an acute hepatopathy. In each of these cases, hospitalization and unmasking for clinical decision making were required. One telmisartan-treated dog developed progressive systemic clinical signs likely associated with *Borrelia burgdorferi* infection, including polyarthopathy, and was removed at study day 63. Arthrocentesis revealed suppurative inflammation and a C6 antibody test was positive. Upon further owner questioning, known tick exposure had occurred one week prior to study inclusion when travelling to Maine. Data from this dog and all other dogs who did not complete the study are included in the intention-to-treat population at all time points prior to removal; data from this dog were not included in the per-protocol population.

Short-term efficacy (Phase I). After 30 days of therapy, median UPC reduction was significantly greater in telmisartan- versus enalapril-treated dogs (-65.3% [-94.9 – 104.0] vs. -34.7% [-74.4 – 86.5]; $P = 0.002$; Figure 3.4].

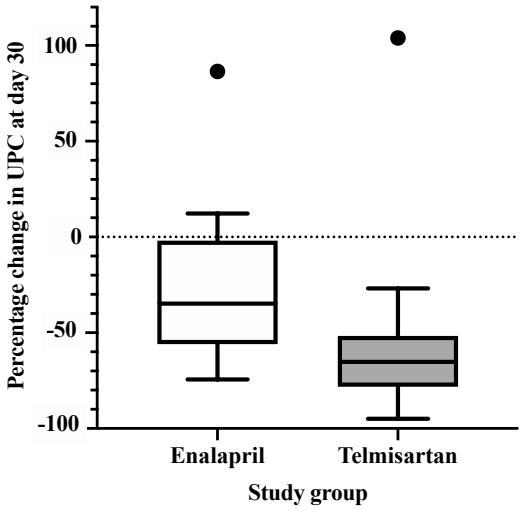

Figure 3.4. Box-plot of percentage change in urinary protein-to-creatinine ratio after 30 days of therapy in 17 dogs receiving enalapril (0.5 mg/kg PO q12h) and 20 dogs receiving telmisartan (1 mg/kg PO in the morning and equal volume of placebo in the evening). Boxes represent the interquartile range and the horizontal bar within each box represents the median. Upper and lower bars and outliers (closed circles) are plotted using the method of Tukey.

Additionally, a greater proportion of telmisartan-treated dogs experienced ≥ 50% UPC reduction after 30 days of therapy (16/20 [80.0%] for telmisartan vs. 6/17 [35.3%] for enalapril; $P = 0.008$). The odds of achieving this endpoint by day 30 was 7.33 times higher in telmisartan-treated, compared to enalapril-treated dogs. There was no significant difference in the proportion of dogs in each treatment group for which UPC at day 30 was ≤ 0.5 (6/20 [30.0%] telmisartan-treated dogs vs. 2/17 [11.8%] enalapril-treated dogs; $P = 0.246$).

Four dogs in the enalapril group and one dog in the telmisartan group (diagnosed with *Borrelia burgdorferi* polyarthropathy during the clinical trial) experienced a UPC increase relative to baseline in the face of therapy.

Intermediate-term efficacy (Phase II). Compared to enalapril, treatment with telmisartan led to a greater median UPC reduction at days 60 (-75.9% [-93.6 – 10.4] vs. -45.2% [-78.4 – 36.2]; $P = 0.016$; Figure 3.5) and 90 (-77.4% [-87.9 – -16.1] vs. -48.8% [-77.2 –79.0]; $P = 0.036$; Figure 3.6). While there was no significant difference in the proportion of dogs achieving the clinical goal of ≥ 50% UPC reduction at days 60 (10/13 [76.9%] for telmisartan vs. 5/12 [41.7%] for enalapril; $P = 0.111$) or 90 (7/8 [87.5%] for telmisartan vs. 4/9 [44.4%] for enalapril; $P = 0.131$), the odds of achieving this endpoint were estimated to be 4.67 and 8.75 times higher with telmisartan than enalapril, by days 60 and 90, respectively. There was no difference in the proportion of dogs attaining a UPC ≤ 0.5 at days 60 (2/13 [15.4%] for telmisartan vs. 2/12 [16.7%] for enalapril; $P = 1.00$) or 90 (1/8 [12.5%] for telmisartan vs. 1/9 [11.1%] for enalapril; $P = 1.00$).

Of the dogs evaluated during phase II, an increase in UPC when compared to baseline was noted at day 60 in two dogs in the enalapril group and one dog in the telmisartan dog despite study drug up-titration. An increase in UPC compared to baseline was documented at day 30 for each of these dogs, as well. At day 90, two enalapril-treated dogs, but no telmisartan-treated dogs, continued to show an increase in UPC relative to baseline.

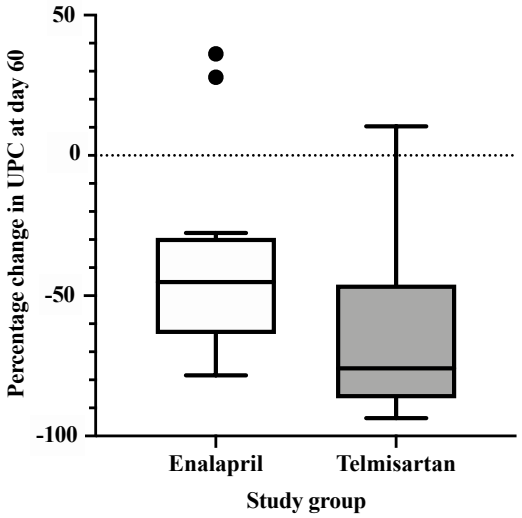

Figure 3.5. Box-plot of percentage change in urinary protein-to-creatinine ratio after 60 days of therapy in 12 dogs receiving enalapril (0.5 mg/kg PO q12h for 30 days, followed by 1.0 mg/kg PO q12h) and 13 dogs receiving telmisartan (1 mg/kg PO in the morning for 30 days followed by 2 mg/kg PO in the morning, equal volume of placebo administered in the evening). Boxes represent the interquartile range and the horizontal bar within each box represents the median. Upper and lower bars and outliers (closed circles) are plotted using the method of Tukey.

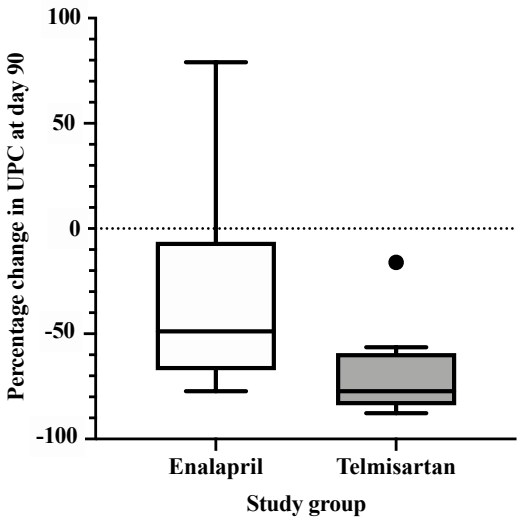

Figure 3.6. Box-plot of percentage change in urinary protein-to-creatinine ratio after 90 days of therapy in 9 dogs receiving increasing dosages of enalapril (1.5 mg/kg PO q12h for the preceding 30 days) and 8 dogs receiving increasing dosage of telmisartan (3 mg/kg PO in the morning and equal volume of placebo in the evening for the preceding 30 days). Boxes represent the interquartile range and the horizontal bar within each box represents the median. Upper and lower bars and outliers (closed circles) are plotted using the method of Tukey.

Per-protocol analyses of short and intermediate-term efficacy. Following enrollment, one telmisartan-treated dog was noted to have fulfilled a criterion for exclusion, after he developed polyarthropathy likely associated with *Borrelia burgdorferi* and known tick exposure in the North East region of the United States of America one week prior to study enrollment. This was the only telmisartan-treated dog to experience an increase in UPC in the face of therapy (i.e., a positive change in UPC) at any time point, and is represented by the outlier data point in figure 3.4. No difference in the results or conclusions of the main efficacy analyses was observed by

censoring this dog's data. On evaluation of the per-protocol population (in which this dog's data are excluded), the significance level for the difference in percentage change in UPC from baseline at days 30 and 60 were $P < 0.001$ and $P = 0.006$, respectively.

Efficacy of combination therapy (Phase III). Combination therapy was performed in 13 (seven enalapril-treated and six telmisartan-treated) dogs, nine of which were followed until day 120. Urinary protein-to-creatinine ratio was numerically lower at day 120 when compared to day 90 in all nine dogs, four of which (one telmisartan group and three enalapril group dogs) achieved a UPC ≤ 0.5. Median percentage change in UPC over the month of combination therapy (i.e., percentage change in UPC from day 90 to day 120), was not significantly different between study groups (-49.9% [-74.4 – -40.8] for telmisartan group vs. -62.4% [-84.0 – -49.3] for enalapril group; $P = 0.519$).

UPC reduction compared to baseline was observed in all 25 dogs who completed the 120-day study period (Figure 3.7). Considering all dogs evaluated at day 120 (regardless of whether they remained on monotherapy or received combination therapy), there was no overall difference in median UPC reduction compared to baseline between the two treatment groups (-84.1% [-95.0 – -22.1] for the telmisartan group vs. -73.9% [-90.4 – -52.7] for the enalapril group; $P = 0.295$; Figure 3.7). There was no difference in the proportion of dogs achieving ≥ 50% UPC reduction between treatment arms (12/13 [92.3%] for telmisartan vs. 12/12 [100.0%] for enalapril; $P = 1.000$). The odds of achieving a ≥ 50% reduction relative to baseline were lower for the telmisartan group. There was also no difference in the proportion of dogs achieving a UPC ≤ 0.5 at day 120 (9/13 [69.2%] for telmisartan vs. 7/12 [58.3%] for enalapril; $P = 0.688$).

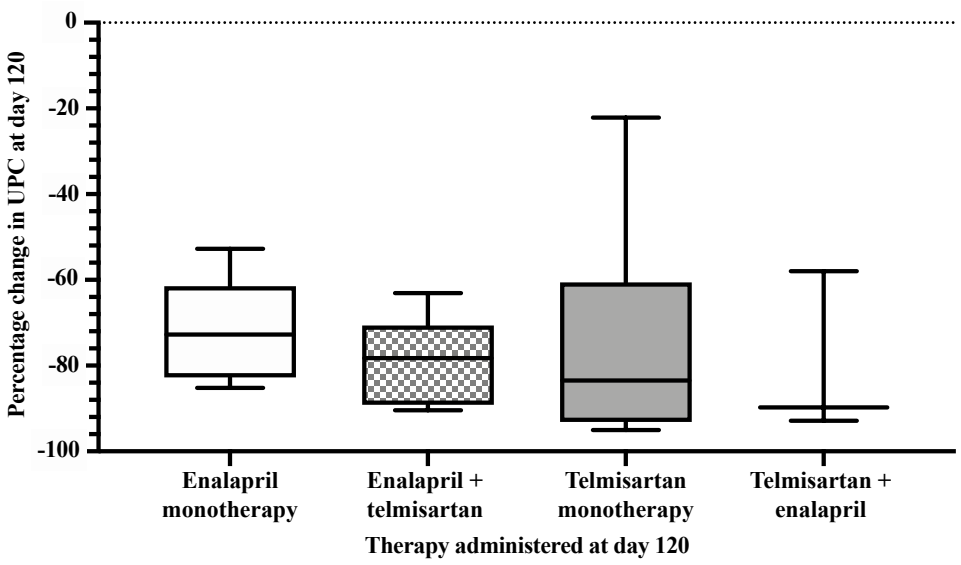

Figure 3.7. Box-plots of percentage change in urinary protein-to-creatinine ratio relative to baseline after 120 days of therapy in proteinuric dogs randomized to receive enalapril (n = 12) or telmisartan (n = 13). Dogs were grouped according to whether they were receiving enalapril (n = 6) or telmisartan (n = 10) as a monotherapy, or combination therapy with a ceiling dose (1.5 mg/kg PO BID) of enalapril plus telmisartan (1.0 mg/kg PO q24h) for the preceding 30 days (n = 6) or with a ceiling dose of telmisartan (3.0 mg/kg PO q24h) plus enalapril (0.5 mg/kg PO BID) for the preceding 30 days (n = 3; line-only). Boxes represent interquartile range and the horizontal bar within each box represents the median. Upper and lower bars and outliers (closed circles) are plotted using the method of Tukey.

Relapse of previously well-controlled proteinuria was noted in one telmisartan-treated dog whose UPC was ≤ 0.5 at day 30 and > 0.5 at day 120, though its UPC reduction was still > 50% compared to baseline by day 120. Similarly, one other telmisartan-treated dog who experienced a UPC nadir corresponding to a 75% reduction by day 60 had its UPC increase at day 120 so that its overall UPC reduction at the end of the study was 22.1%.

Time to study endpoints. Using the up-titration scheme defined here, dogs treated with telmisartan achieved the clinical endpoint of ≥ 50% UPC reduction significantly faster than those treated with enalapril (P = 0.020; Figure 3.8). There was no significant difference in time to achieve the clinical endpoint of UPC ≤ 0.5 between study groups.

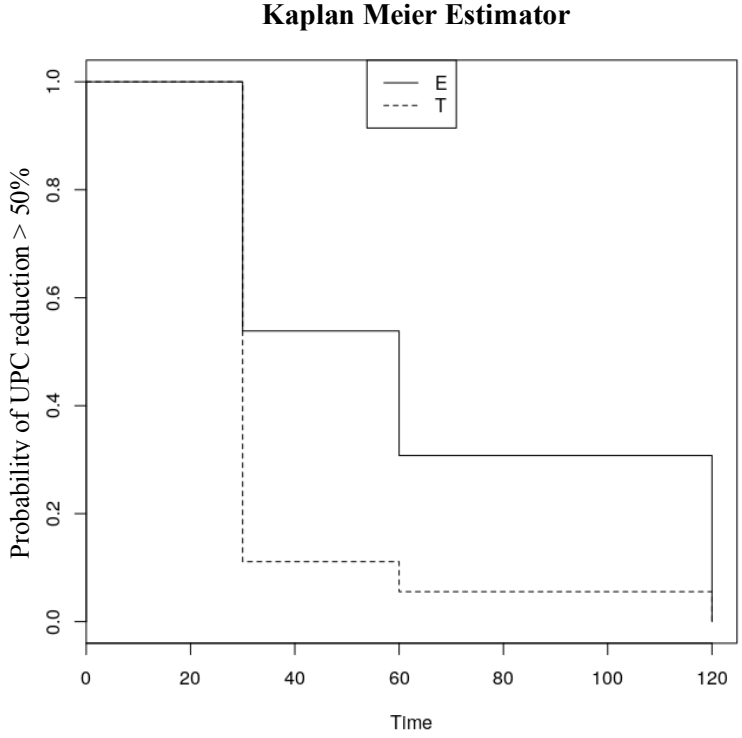

Figure 3.8. Kaplan Meier plot for time to ≥ 50% reduction in urinary protein-to-creatinine ratio (UPC), when compared to baseline, in dogs treated with increasing dosages of telmisartan or enalapril, or a combination of the two. Solid line represents dogs treated with enalapril. Dotted line represents dogs treated with telmisartan.

Antihypertensive therapy. A total of 10 (six enalapril-treated and four telmisartan-treated) dogs received amlodipine during the study period. One dog in each treatment group was receiving amlodipine prior to enrollment (Table 3.3). The median (range) maximum dosage of

amlodipine administered to telmisartan-treated and enalapril-treated dogs was 0.205 (0.09-0.35) mg/kg/day and 0.265 (0.11-0.38) mg/kg/day, respectively. The median final dosage (i.e., dosage being administered at the end of study participation) was the same as the maximum dosage for the enalapril group. For the telmisartan group, median final dosage of amlodipine was 0.13 (0.09-0.35) mg/kg/day.

For dogs not receiving amlodipine, a significantly greater mean percentage reduction in SBP at day 30 was observed in the telmisartan group (-12 ± 9%) when compared with the enalapril group (-1 ± 12%; $P = 0.008$).

Hypotension, as defined in the present study (i.e. <100 mmHg in a dog with clinical signs of hypotension), was not observed at any visit. In one telmisartan-treated dog not receiving amlodipine, average SBP was 98 mmHg at day 30; however, the owners of this dog reported signs of increased energy compared to baseline. Eleven telmisartan- (including the above-mentioned dog) and three enalapril-treated dogs had SBP measurements between 100 and 120 mmHg documented in one or multiple visits. One dog in each group with SBP 100-120 mmHg was concurrently receiving amlodipine, which was initiated prior to enrollment.

Safety analyses. After 30 days of therapy, there was no difference in percentage change in Cr ($P = 0.278$), K ($P = 0.419$) or Hct ($P = 0.057$) between treatment groups (Figure 3.9). At one-week recheck following increase to the 'ceiling dosages' of the study medications (i.e., day 67±1), significantly greater percentage Hct reduction compared to baseline was observed in dogs treated with telmisartan versus those treated with enalapril (-9 ± 10% for telmisartan vs. -3 ± 15%; $P = 0.026$; Figure 3.10). No difference in percentage change in Cr ($P = 0.96$) or K ($P = 0.757$) was observed at the same timepoint.

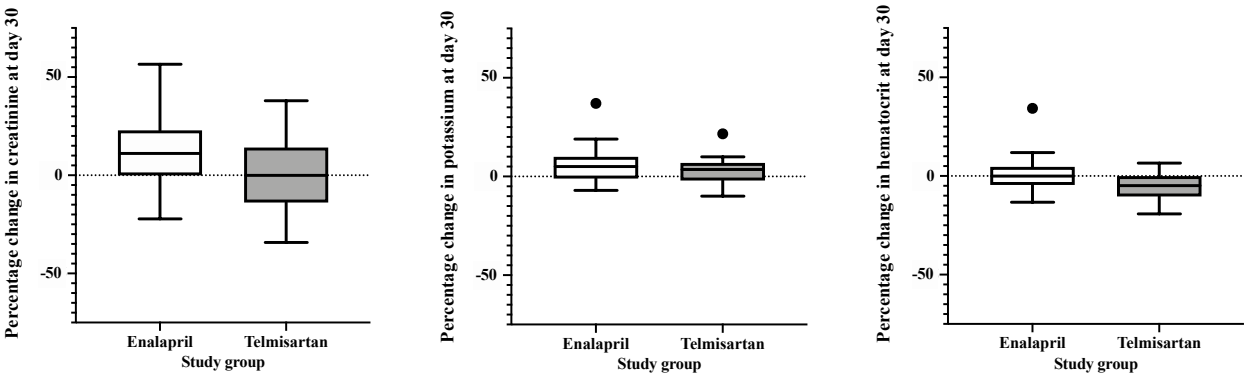

Figure 3.9. Box-plot of percentage change in blood creatinine concentration, blood potassium concentration and hematocrit at day 30, when compared to baseline values, in 17 enalapril-treated and 20 telmisartan-treated proteinuric dogs. Boxes represent the interquartile range and the horizontal bar within each box represents the median. Upper and lower bars and outliers (closed circles) are plotted using the method of Tukey.

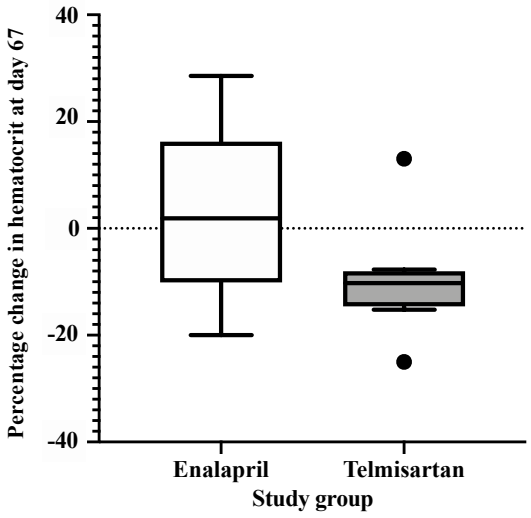

Figure 3.10. Box-plot of percentage change in hematocrit at day 67, when compared to baseline values, in nine enalapril-treated and nine telmisartan-treated proteinuric dogs. Boxes represent the interquartile range and the horizontal bar within each box represents the median. Upper and lower bars and outliers (closed circles) are plotted using the method of Tukey.

When considering only the first 90 days of therapy, 2 (10.0%) of 20 dogs treated with telmisartan and 4 (21.1%) of 19 dogs treated with enalapril as a monotherapy experienced an increase in Cr that prompted removal from the study.

Thirteen dogs received combination therapy during the last 30 days of treatment (days 90 through 120). Of these, seven dogs were initially assigned to the enalapril group and were, therefore, receiving 1.5 mg of enalapril /kg q12h and 1 mg of telmisartan /kg q24h. The remaining six dogs were assigned to the telmisartan group and were consequently receiving 3 mg of telmisartan/kg q24h and 0.5 mg of enalapril /kg q12h. Four dogs, three in the telmisartan group and one in the enalapril group, experienced azotemia requiring removal from the study after the other study drug was added to 'ceiling dosages' of the assigned initial medication. Blood creatinine returned to each individual's baseline value following temporary discontinuation of all RAAS blockers; however, hospitalization and in-patient treatment was required for one of these dogs, who suffered acute kidney injury.

Owner reported adverse events. Owner-reported adverse events occurring during the study period are summarized in table 3.4. With two exceptions, reported changes were mild and no alterations in demeanor, appetite, activity or any combination of these, were by themselves a cause for early removal from the study (i.e., all dogs removed from the present study had met one of the safety endpoints or developed a significant concurrent disease, requiring unmasking for clinical management). In one case described in "Protocol adherence and completion of study period" above, lethargy and anorexia developing following combination therapy were severe enough to prompt discontinuation of study medications. Similar clinical signs were observed in another dog, also receiving combination therapy, who developed acute kidney injury on dual

RAAS blockade. Increased activity and improved general well-being were reported by the owners of two enalapril-treated, one combination-treated and five telmisartan-treated dogs.

Table 3.4. Summary of owner reported adverse events from telmisartan-treated (n = 20) and enalapril-treated (n = 19) dogs. Data are presented as number of dogs for which each event was reported.

Adverse events		Enalapril monotherapy	Telmisartan monotherapy	Dual RAAS blockade
Activity	Lethargy / decreased activity	2	2	1
	Somnolence	1	1	0
	Irritability	1	0	0
Appetite	Inappetence	7	6	7
	Increased appetite	2	0	0
Gastrointestinal	Vomiting	5	5	1
	Diarrhea	3	7	1
Urinary	Polyuria/polydipsia	2	0	0
	Pollakiuria	2	0	0
	Urinary incontinence	2	1	0
	Nycturia	0	1	0
	Pigmenturia	0	1	0
	Decreased urination	0	1	0
	Urinary tract infection	0	2	0
Respiratory	Cough	1	1	0
	Reverse sneezing	1	0	0
Dermatologic	Pruritus	1	0	0
	Aural hematoma	1	0	0
	Acute moist dermatitis	0	1	0
Other	Corneal ulcer	1	0	0
	Tooth root abscess	1	0	0
	Ataxia	1	0	0
	Lameness	0	1	0
	Vestibular disease	0	1	0

Discussion

The present study showed that, compared to a standard dose of enalapril, treatment with telmisartan led to both a greater median UPC reduction and a greater proportion of dogs achieving a 50% UPC reduction at day 30. Although data from the intermediate-term analyses may be biased towards dogs with worse response to therapy, as the dogs with clinically controlled proteinuria were not evaluated until the end of the study (day 120), telmisartan's antiproteinuric effects remained superior to those of enalapril, at the dosages studied, at days 60 and 90. Furthermore, with the exception of one dog that was later found to be developing an active response to systemic infectious disease, treatment with telmisartan resulted in a reduction in UPC in all dogs who received it, whereas four enalapril-treated dogs experienced an increase in UPC in the face of ACE inhibition. In a multicenter, masked, placebo-controlled clinical trial designed to evaluate the efficacy of enalapril, administered at a dosage of 0.5 mg/kg PO q12-24h for 6 months, as treatment for naturally occurring proteinuria, a clinically significant improvement was noted in 9 (56%) of 16 enalapril-treated subjects.[143] In the referenced study, clinically significant improvement was defined for dogs with $\geq$ 50% UPC reduction and stable serum creatinine. In the same study, no progression of disease (i.e., <50% reduction in UPC with stable serum creatinine) was observed in 4 (25%) of 16 enalapril-treated dogs, whereas 3 (18.8%) of the dogs experienced disease progression (i.e., >50% increase in UPC, serum creatinine, or both) despite therapy with enalapril.[143] In the present study, although the follow-up period was shorter, $\geq$ 50% UPC reduction was observed in only 6/17 (35.3%), 5/12 (41.7%) and 4/9 (44.4%) enalapril-treated dogs evaluated at days 30, 60 and 90, respectively. Therefore, a clinically relevant change in UPC was noted in the minority of enalapril-treated dogs during the monotherapy phase of the study. Conversely, $\geq$ 50% UPC reduction occurred in the majority of

telmisartan-treated dogs at all time points evaluated. Additionally, the odds of achieving a clinically relevant reduction were consistently higher for telmisartan-treated dogs in the monotherapy phases of this study.

In a previous study of healthy dogs, telmisartan led to significantly greater attenuation of the pressor response to exogenous AngI when compared to placebo, enalapril and losartan.[162] Data from that pre-clinical study and the present clinical trial suggest that telmisartan achieves superior RAAS blockade than does the ACEi enalapril in this species. Similar observations have been made in studies evaluating human beings. Weighted evidence from a meta-analysis reviewing data from 20 randomized controlled trials that collectively studied over 25,000 people and compared the efficacy of telmisartan to those of placebo, an ACEi, a different ARB, non-RAAS-blocking antihypertensive drug therapy, or no medication, favored telmisartan's efficacy for the improvement of, and prevention of progression of, proteinuria or albuminuria, both overall and within comparison categories.[160] While one veterinary case report has described the successful use of telmisartan for the treatment of a dog with refractory proteinuria,[163] systematic evidence regarding the efficacy of any ARB for the treatment of proteinuria has been lacking prior to the present study.

Proteinuria is proposed to promote renal injury through several mechanisms,[64, 131] and is a well-documented risk factor for uremic crises, and renal-related and all-cause mortality in dogs.[140-142] In accordance with the guidelines for treatment of canine proteinuria set forth by the American College of Veterinary Internal Medicine,[146, 147] the therapeutic target of the present study was UPC $\leq$ 0.5. Further, at the time of study design, combination therapy with ACEi and ARBs was common practice in human medicine and reported to be associated with beneficial hemodynamic effects.[236-240] In addition to evaluating monotherapy, the present study was also

designed to evaluate the efficacy of the combination of enalapril and telmisartan in dogs with UPC > 0.5 on 'ceiling dosages' of either of these medications. Since the start of this trial, several studies and meta-analyses evaluating human beings report that although efficacy is potentially improved when these classes are combined, dual RAAS inhibition has failed to improve cardiovascular or renal outcomes, and increases the risk of adverse events.[159, 241, 242] Consequently, combination therapy with an ACEi and ARB is no longer a general recommendation for human renal or cardiac disease. The findings of the present study are in accordance with such cautionary studies in human beings. Dual RAAS blockade did lead to UPC reduction in all dogs with proteinuria refractory to monotherapy, with 4 (30.8%) of the 13 combination-treated dogs achieving UPC ≤ 0.5; however, this practice was associated with the development of clinically relevant azotemia within 7 days in a relatively high percentage (30.8%) of these 13 dogs. One of the four dogs that developed azotemia required hospitalization for treatment of suspected RAAS blocker-induced acute kidney injury.[243] The authors therefore advise caution when combining these medications, as a greater proportion of dogs developed azotemia following initiation of therapy with an ACEi/ARB combination than following up-titration of either medication when used as a single agent. However, it deserves comment that in the present study, dual therapy was performed by combining maximum dosages of one of the RAAS blockers with a starting dosage of the other. In human beings, combination of these medications at low dosages was deemed safe and efficacious at reducing proteinuria in patients with advanced CKD.[238] Whether that would be true in dogs remains to be studied.

The development of azotemia, hyperkalemia, or even acute kidney injury is a well-described potential adverse effect of RAAS blockade in patients with renal or cardiac disease.[244] Ten dogs were removed from the present study prior to day 120 due to the development of

azotemia and, in one case, substantial hyperkalemia (i.e., K> 6.5 mmol/L). There was no difference in the percentage change in Cr or K between study groups at any monotherapy recheck (i.e., days 30 or 67). However, these data are biased towards dogs who did not experience a significant change in these parameters, as dogs who did would have been removed from the study. There was also no difference in the overall proportion of dogs removed from each study group, although two dogs in the enalapril group, versus no dogs in the telmisartan group, were removed within 14 days of starting therapy. Unlike telmisartan which undergoes biliary excretion into the feces as its predominant route of elimination,[245] enalapril's main route of elimination is via renal excretion.[246] In dogs with renal insufficiency, accumulation of its active metabolite, enalaprilat, and increased ACE inhibition may occur, modifying the relationship between drug dosage and its effect.[247]

The present study was not designed specifically to assess the relative BP-lowering effects of telmisartan and enalapril. Nonetheless, when patients receiving amlodipine (i.e., dogs with SBP > 180 and those already receiving the drug) were excluded from analysis, compared to enalapril-treated dogs, telmisartan-treated dogs experienced a greater SBP reduction within the first 30 days of treatment. In cats, two large multicenter, randomized, masked, placebo-controlled clinical trials recently documented the antihypertensive efficacy of telmisartan compared to placebo.[213, 214] Clinical data regarding telmisartan's efficacy as an antihypertensive in dogs is limited to that of a single case series describing its use in five dogs with refractory systemic hypertension.[174] Prospective clinical research in spontaneously hypertensive dogs is warranted to explore this drug's potential as an antihypertensive in this species.

Angiotensin II has far-reaching effects. In addition to its commonly cited role in the regulation of blood pressure, electrolyte and fluid balance, AngII is also a modulator of

erythropoeiesis.[248, 249] Angiotensin II signaling regulates renal transcriptional activation of erythropoeietin,[248] increasing proliferation of early erythroid progenitors.[250] Both ARBs and ACEi are known, on rare occasion, to cause severe anemia associated with impaired erythropoiesis in human beings.[251] In the present study, dogs treated with telmisartan experienced a significantly greater, though rarely clinically significant, decrease in hematocrit over 67 days, when compared to those treated with enalapril. As the stimulatory effects of AngII on erythropoiesis appear to be mediated via the AT_1R,[249, 250] it is possible that telmisartan achieved more complete RAAS blockade, having a larger impact on red blood cell production. Measurement of serum erythropoietin concentration was not performed in the present study; however, it would be recommended for further dissection of the impact of RAAS blockers on red blood cell hemostasis in dogs.

A main limitation of our study is the lack of a specific histologic diagnosis for the cause of proteinuria, as renal biopsy was not pursued in our patients. Immune-complex glomerulonephritis was reported in nearly half (48.1%) of the cases included in a large retrospective study of canine renal biopsy specimens obtained for suspected glomerular disease.[136] Therefore, it is likely that this condition, which may be less responsive to RAAS inhibition that non-immune-mediated causes, affected a subset of our study population. Nonetheless, treatment randomization makes it less likely that these dogs were disproportionally assigned to a study group. A second limitation is the lack of a complete set of monthly recheck data for dogs who reached the study endpoint (i.e., UPC $\leq$ 0.5) prior to day 120. Because those dogs were not re-evaluated until day 120, direct comparisons of median UPC reduction at days 60 and 90 is biased towards "non-responder" dogs, in which proteinuria was not clinically controlled. Disproportionate removal of dogs who met a safety endpoint or who suffered from a

seemingly unrelated illness that prompted investigator and owner unmasking, may also have introduced bias in these data. Additionally, for dogs whose proteinuria was seemingly transiently controlled, time between control and clinical relapse was uncharacterized.

In conclusion, compared to enalapril, telmisartan treatment led to a greater percentage reduction in UPC in the short- and intermediate-term, and produced a clinically relevant UPC reduction (i.e., ≥ 50% reduction) in a greater proportion of dogs and at a faster rate. No difference in the safety profiles of these medications was observed. These data support the superiority of telmisartan when compared to enalapril as previously documented in pre-clinical canine studies, and suggest that this ARB may show promise as a first-line RAAS blocker for treatment of canine renal proteinuria. Larger studies evaluating dogs with a histologic diagnosis for their proteinuric disease are warranted for further investigation of telmisartan's antiproteinuric effects.

Footnotes

a. SNAP Heartworm RT Test, IDEXX Laboratories, Westbrook, ME.

b. SNAP 4Dx Plus, IDEXX Laboratories, Westbrook, ME.

c. Semintra, Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany.

d. Enalapril, Taro Pharmaceuticals Industries Ltd., or Valeant Pharmaceuticals International, Inc., USA, 20 mg tablets compounded into a suspension by the UGA Veterinary Teaching Hospital Pharmacy.

e. R Development Core Team, Vienna, Austria.

f. GraphPad Prism for Mac, version 7, GraphPad Software Inc, La Jolla, CA.

CHAPTER 4

PRO-FIBROTIC GENE TRANSCRIPTION IN AN ISCHEMIC MODEL OF FELINE CHRONIC KIDNEY DISEASE[2]

[2] Bianca N. Lourenço, Amanda E. Coleman, Chad W. Schmiedt, Cathy A. Brown, Daniel R. Rissi, James B. Stanton, Steeve Giguère, Roy D. Berghaus, Scott A. Brown, and Jaime L. Tarigo. Submitted to the American *Journal of Veterinary Research*, 05/01/2019.

Abstract

Objective: To characterize the transcription of hypoxia-induced mediators of fibrosis in renal tissues from cats with experimental chronic kidney disease (CKD).

Samples: Banked renal tissues from six purpose-bred cats that underwent transient unilateral renal ischemia as a model of CKD six months previously (RI group) and from eight healthy control cats.

Procedures: Tissues from both the ischemic and contralateral kidneys of RI group cats, and from the right kidney of healthy control cats were assessed histologically for fibrosis, inflammation, and tubular atrophy, and by reverse transcription-quantitative polymerase chain reaction to characterize the transcript levels of hypoxia-inducible factor (HIF)-1α, matrix metalloproteinases (MMP)-2, -7 and -9, tissue inhibitor of metalloproteinase (TIMP)-1, transforming growth factor (TGF)-β1, and vascular endothelial growth factor (VEGF)-A. Gene transcription was compared among kidney groups, and the correlation between transcript levels and histologic scores of the ischemic kidneys was investigated.

Results: The MMPs, TIMP-1, and TGF-β1 were significantly upregulated in tissues from the RI group: MMPs and TGF-β1 in the ischemic, and TIMP-1 in both the ischemic and contralateral kidneys. VEGF-A was bilaterally downregulated in the CKD model. Transcription of HIF-1α was not different among kidney groups. Transcript levels of HIF-1α, MMP-2, MMP-7, and TIMP-1 were positively correlated with fibrosis scores in the ischemic kidneys.

Conclusions and Clinical Relevance: Alterations in the renal transcription of genes involved in pro-fibrotic pathways were noted bilaterally six months following a single event of unilateral renal ischemia, suggesting that one episode of unilateral renal injury may have a lasting impact on both kidneys.

Abbreviations

ABL, v-abl Abelson murine leukemia viral oncogene homolog

CKD, Chronic kidney disease

ECM, Extracellular matrix

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

HIF-1α, Hypoxia-inducible factor-1α

MMP-2, Matrix metalloproteinase-2

MMP-7, Matrix metalloproteinase-7

MMP-9, Matrix metalloproteinase-9

NCBI, National Center for Biotechnology Information

RI, Renal ischemia

RPS7, Ribosomal protein S7

TGF-β1, Transforming growth factor-β1

TIMP-1, Tissue inhibitor of metalloproteinase-1

VEGF-A, Vascular endothelial growth factor-A

Introduction

Chronic kidney disease is common in aged domestic cats, with prevalence estimates ranging up to 30–50%.[5, 8] Histologically, feline CKD is characterized by tubulointerstitial fibrosis,[252] a finding that is strongly correlated with functional impairment.[185, 253]

Our group recently reported a model of feline CKD that utilized transient, unilateral, *in vivo* renal ischemia to induce chronic fibrosis, interstitial inflammation, and tubular atrophy.[200, 225] As similar lesions are present in cats with naturally occurring CKD,[183, 252] the results of those

studies further support accumulating evidence that emphasizes the critical role of renal hypoxia in the intrinsic progression of CKD.[32, 254, 255]

Increased genetic expression of hypoxia-induced mediators of fibrosis has been demonstrated in CKD of non-feline species;[32, 255] however, the molecular pathways leading to renal fibrosis in cats are poorly described. The hypoxia-inducible factor family plays a major role in the regulation of adaptive responses to renal hypoxia.[256, 257] While its role in CKD remains to be fully elucidated, HIF-1α has been suggested as a critical factor for the development of renal fibrosis in hypoxic conditions and subsequent renal failure in mice.[258] Transforming growth factor-β1,[112] as well as matrix metalloproteinases and their tissue inhibitors, appear to be involved in CKD by modulating ECM composition.[105, 226] While increased urinary TGF-β1 levels have been documented in cats with naturally occurring CKD,[204] expression of MMPs and TIMPs in feline renal disease has not been characterized. Vascular endothelial growth factor, a proliferative, survival, and trophic factor for endothelial cells, is also strongly stimulated by hypoxia;[116] however, the limited data available from cats with naturally occurring CKD conflict with regards to its role in this disease.[a,205]

The objective of this study was to characterize the transcript levels of hypoxia-induced mediators of fibrosis in ischemia-induced feline CKD using reverse transcription-quantitative polymerase chain reaction analysis. We hypothesized that, compared to tissues from healthy controls, those from affected cats would demonstrate altered transcription, and that the degree of transcription of these factors would be correlated with severity of renal histopathologic changes in the previously ischemic kidneys.

Materials and methods

Samples. This study utilized banked renal tissue samples collected from two populations of domestic cats: 1) cats having previously undergone transient, unilateral, *in vivo* renal ischemia as a model of renal fibrosis and CKD (RI group, n = 6), and 2) healthy control cats without evidence of CKD (control group, n = 8). Tissues from the RI group were obtained from purpose-bred female spayed cats that completed a prior study assessing the chronic effects of unilateral, 90-minute renal ischemia on renal function and histology.[200] Briefly, these cats underwent general anesthesia for temporary occlusion of the renal artery and vein of the right kidney (i.e., the "ischemic kidney"), via midline laparotomy. The left kidney (i.e., the "contralateral kidney") was undisturbed during this procedure. Cats were recovered from anesthesia and followed for six months, after which they were humanely euthanized for purposes unrelated to the present study. Renal samples were collected and banked at necropsy. Tissues from control cats were obtained from the right kidney of purpose-bred, clinically healthy, mixed breed, intact female cats that were previously enrolled in unrelated terminal studies. The University of Georgia's Institutional Animal Care and Use Committee approved all activities related to these studies.

Assessment of renal function. For the RI group cats, data reflecting renal function, including glomerular filtration rate, serum concentrations of urea nitrogen and creatinine, urine specific gravity and urinary protein-to-creatinine ratio were collected throughout the original study.[200] Biochemical data was obtained in two separate occasions within four days of euthanasia, and mean values from these two measurements are reported here. For the control group cats, renal biochemistry, urinalysis and urinary protein-to-creatinine ratio measurement were performed the day of euthanasia. These cats were deemed to have normal renal function and structure on the basis of biochemical serum and urine analyses [i.e., serum creatinine <1.6

mg/dL (< 140 μmol/L), urine specific gravity > 1.035, and urinary protein-to-creatinine ratio ≤ 0.2] and evaluation of renal histology. All clinical pathology analyses were performed by the Clinical Pathology Laboratory of the University of Georgia College of Veterinary Medicine.

Tissue handling and processing. Immediately following euthanasia, kidneys were removed through a midline laparotomy and sectioned longitudinally. One-fourth to one-half of each kidney was minced and placed in RNA stabilization solution,[b] and the remaining portion was placed in neutral-buffered 10% formalin. In this way, equal proportions of renal cortex, medulla and corticomedullary junction were maintained for each sample. Following overnight incubation at 4°C, tissues were removed from RNA stabilization solution, homogenized with a mortar and pestle, divided into 30 mg aliquots, and stored at -80°C until further analysis.

Reverse transcription and quantitative polymerase chain reaction. Total RNA was extracted from 30 mg of tissue for each sample using a commercially available RNA extraction kit,[c] and quantified using a spectrophotometer.[d] The integrity of the isolated RNA was confirmed first by the visualization of 18S and 28S ribosomal bands on 1.2% agarose gels, followed by analysis using a bioanalyzer system.[e]

For each sample, 1 μg of RNA was treated with deoxyribonuclease,[f] and reverse-transcribed using a cDNA reaction master mix.[g] Quantification was performed in 20 μL reactions containing 10 μL of SYBR Green Supermix,[h] 5 pmol of each primer (Table 4.1), and 9 μL of complementary DNA sample at a 1:40 dilution. Thermal cycling conditions[i] consisted of an activation step at 95°C for 30 seconds, followed by 36 amplification cycles (95°C for 15 seconds for denaturation and 60°C for 30 seconds for annealing and extension), and a melt curve step (60°C to 95°C, increasing at increments of 0.5°C every 5 seconds). All reactions were performed in triplicate and average values were used for further analyses. A no reverse transcriptase control

was included for each sample and three no template controls were used in each plate. In accordance with the sample maximization method,[259] all samples were analyzed in one 96-well plate and each gene was analyzed in a single run. A standard sample was analyzed in triplicate with GAPDH primers in each plate and used as an inter-run control for the entire gene study.

Table 4.1. Primer sequences for quantitative polymerase chain reaction.

Gene	Ensemble ID/ NCBI Access. No.	Primer sequence (5'→ 3')	Fragment size (bp)	Source
GAPDH	ENSFCAG 00000006874	for: GCTGCCCAGAACATCATCC rev: GTCAGATCCACGACGGACAC	134	Previously reported[260]
RPS7	NM_001009832	for: GTCCCAGAAGCCGCACTT T rev: CACAATCTCGCTCGGGAA AA	74	Previously reported[261]
ABL	ENSFCAT 00000005306	for: TGTGGCGAGTGGTGATAATACAC rev: TCCACTCACCATTCTGGTTGTAA	83	Previously reported[261]
HIF-1α	XM_001493206	for: TTGGCAGCAATGACACAGACACTG rev: TTGAGTGCAGGGTCAGCACTACTT	175	Previously reported[262]
MMP-2	XM_003998042.2	for: AGACAAGTTCTGGAGGTACAATG rev: CGCCCTTGAAGAAGTAGCTGT	149	NCBI Primer-BLAST
MMP-7	XM_003992303.2	for: CTTTGCAAGAGGAGCTCACG rev: AATTCCTAGACCCCTGCCGT	148	NCBI Primer-BLAST
MMP-9	XM_003983412.4	for: GCTTCTGGAGGTTCGACGTG rev: CAATAGAAGCGGTCCTGGCA	148	NICBI Primer-BLAST
TGF-β1	AY425617.1	for: AGCACGTGGAGCTGTACCAGAAAT rev: TCCAGTGACATCAAAGGACAGCCA	110	Previously reported[263]
TIMP-1	XM_011291721.2	for: TCTCATCGCCGGAAAACTGC rev: AGCCAGCAGCATAGGTCTTG	122	NCBI Primer-BLAST
VEGF-A	AB071947.1	for: TTTCTGCTCTCTTGGGTGCATTGG rev: TGCGCTGGTAGACATCCATGAACT	139	Previously reported[262]

Transcript levels of the target genes HIF-1α, MMP-2, MMP-7, MMP-9, TGF-β1, TIMP-1 and VEGF-A were normalized to those of three reference genes, GAPDH, RPS7 and ABL, using

the GENorm method.[264] Reference genes were selected based on their primer efficiency, stability value (M value < 0.5) and coefficient of variance (< 0.25) when tested in a complete set of the experiment's samples. After normalization, for each target gene transcript levels were scaled to those of the lowest sample.

Gene specific primers were selected from previously reported studies, when available, or designed using the NCBI primer-BLAST[265] (Table 4.1). Primer efficiencies were calculated using the Pfaffl method based on standard curves performed with serial dilutions of cDNA (factor of two; three replicates) starting at a 1:20 dilution. Quantitative PCR products were confirmed on 1.2% agarose gels. Amplicons were cloned into a vector system[j] and sequenced bi-directionally by chain termination.[k] Resulting sequences were confirmed via NCBI-BLAST against the feline genome.

Histology. Three-micron thick sections of paraffin-embedded, formalin-fixed tissues were stained with Masson's trichrome, hematoxylin and eosin, and periodic acid-Schiff and hematoxylin. Ten consecutive 20× fields in each of the cortex and corticomedullary junction were scored for degree of fibrosis, inflammation, and tubular atrophy by two board-certified veterinary pathologists, as previously described.[200] Briefly, degree of fibrosis was evaluated in Masson's trichrome stained sections, inflammation was assessed in hematoxylin and eosin stained slides, and tubular atrophy was scored in periodic acid-Schiff and hematoxylin stained sections. The grading scheme for each histologic score ranged from 0 (absent) to 3 (severe) (Table 4.2). Numeric scores of the 20 (10 cortical and 10 corticomedullary) examined fields were averaged to generate mean scores for inflammation, tubular atrophy, and fibrosis for each kidney. A previous report[200] and figure 4.1 provide examples of sections illustrating each histologic grade.

Table 4.2. Scoring system for inflammation, tubular atrophy and fibrosis evaluated on histology.

Histologic scores			
Score	Inflammation	Tubular atrophy	Fibrosis
0	No inflammatory cells	No atrophy	Absent, no increase compared to normal
1	Mild, < 10% of interstitium affected	Mild, fewer than 10 scattered atrophic tubules	Mild, rare foci/segments of fibrosis involving < 20% of cortex
2	Moderate, 20-50% of field affected	Moderate, linear streaks of tubular atrophy often with fibrosis and inflammation	Moderate, fibrotic segments involving 20 – 30% of cortex
3	Severe, > 50% affected	Severe, two or more streaks of tubular atrophy	Severe, fibrotic segments involving > 30% of cortex

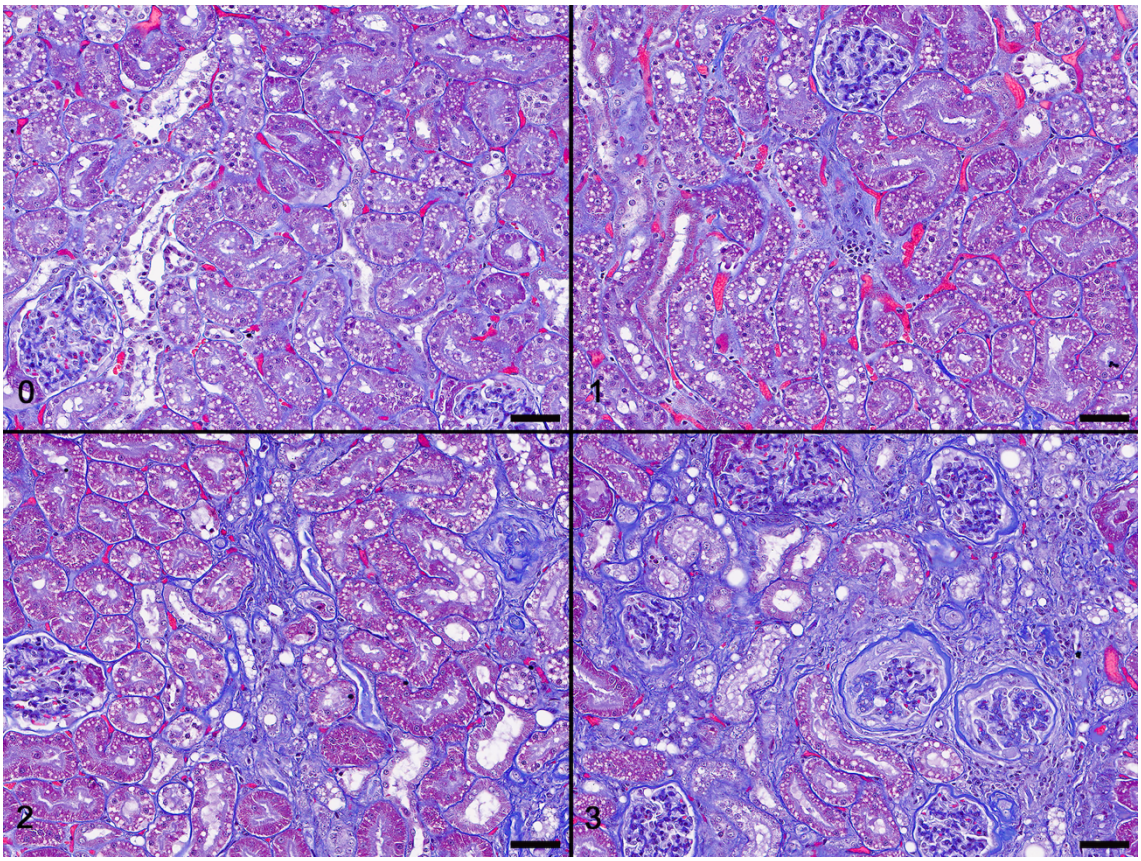

Figure 4.1. Example images of each possible fibrosis score. 0, absent; 1, mild; 2, moderate; and 3, severe (Masson's trichrome-stained sections; 20x; scale bar represents 100 µm).

Statistical analyses. Commercial software packages[l,m] were used for statistical analyses. Normality of data was assessed based on visual examination of histograms and normal Q-Q plots of the residuals. Clinical and demographic data of cats from the RI and control groups were compared using the Mann-Whitney U test. For the purposes of comparison, urine specific gravity measurements > 1.060 were assigned a value of 1.061.

Normalized gene transcript levels were transformed to the natural logarithm and analyzed using linear mixed-effects modeling with cat modeled as a random effect to account for the correlated structure of the data (i.e., accounting for the fact that pairs of ischemic and contralateral kidneys from RI group cats were evaluated), and kidney modeled as a fixed nominal effect. Model fit was assessed using Akaike information criterion values. Values of *P* were adjusted for multiple comparisons using the method of Šídák. For the ischemic kidneys, associations between histology scores and the transcript levels of each gene were investigated using the Spearman rank correlation coefficient. For all analyses, values of $P < 0.05$ were considered statistically significant.

Results

Animals. Clinical characteristics of the cats from which the banked renal tissues originated are listed in Table 4.3. Cats that had undergone unilateral renal ischemia were, as a population, older ($P = 0.002$) and heavier ($P = 0.008$) than control cats. Cats of the RI group had a progressive decline in glomerular filtration rate and increase in serum creatinine concentration over the course of the original 6-month study.[200]

Table 4.3. Clinical and laboratory data from cats who underwent renal ischemia as a model of chronic kidney disease six months prior (n = 6) and from healthy control cats (n = 8). For the renal ischemia group, values for biochemical variables represent the average of two measurements obtained within four days of euthanasia. Numerical data are presented as median (range).

	Renal ischemia group	Control group	Laboratory reference interval
Number	6	8	
Age (days)*	520 (472-588)	268 (228-294)	
Sex*	Female spayed	Female intact	
Body weight (kg)*	4.72 (3.93-5.35)	3.59 (2.29-4.47)	
Serum creatinine concentration*	1.4 (1.3-1.6) mg/dL 124 (115-141) µmol/L	1.0 (0.8-1.2) mg/dL 88 (70-106) µmol/L	0.6-1.8 mg/dL 53-159 µmol/L
Serum urea nitrogen concentration	24 (22-27) mg/dL 8.6 (7.9-9.6) mmol/L	27 (23-34) mg/dL 9.6 (8.2-12.1) mmol/L	21 – 36 mg/dL 7.5-12.9 mmol/L
Urine specific gravity	1.056 (1.041-1.059)	1.055 (1.037-1.061†)	N/A
Urinary protein-to-creatinine ratio*	0.1 (0.05-0.11)	0.14 (0.1-0.2)	< 0.5

* Variables differ significantly between groups ($P < 0.01$).

† Urine specific gravity measurements with results described as > 1.060 were assigned a value of 1.061, for comparison purposes.

Normalized gene transcript levels. Transcript levels were evaluated in all tissues, with the exception of one contralateral kidney sample of the RI group for which RNA quality was insufficient. Statistically significant differences in the normalized transcript levels of MMP-2, MMP-7, MMP-9, TIMP-1, TGF-β1, and VEGF-A were observed among the three kidney groups (Figure 1). The levels of MMP-2 transcripts were significantly higher in ischemic compared to contralateral kidneys ($P = 0.008$), but not compared to control kidneys. Transcripts of MMP-7 and -9 were significantly more abundant in ischemic compared to both contralateral and control kidneys ($P < 0.001$ and $P = 0.002$, respectively). Transcription of TGF-β1 was significantly

increased in ischemic compared to control kidneys ($P = 0.045$), but not ischemic compared to contralateral kidneys.

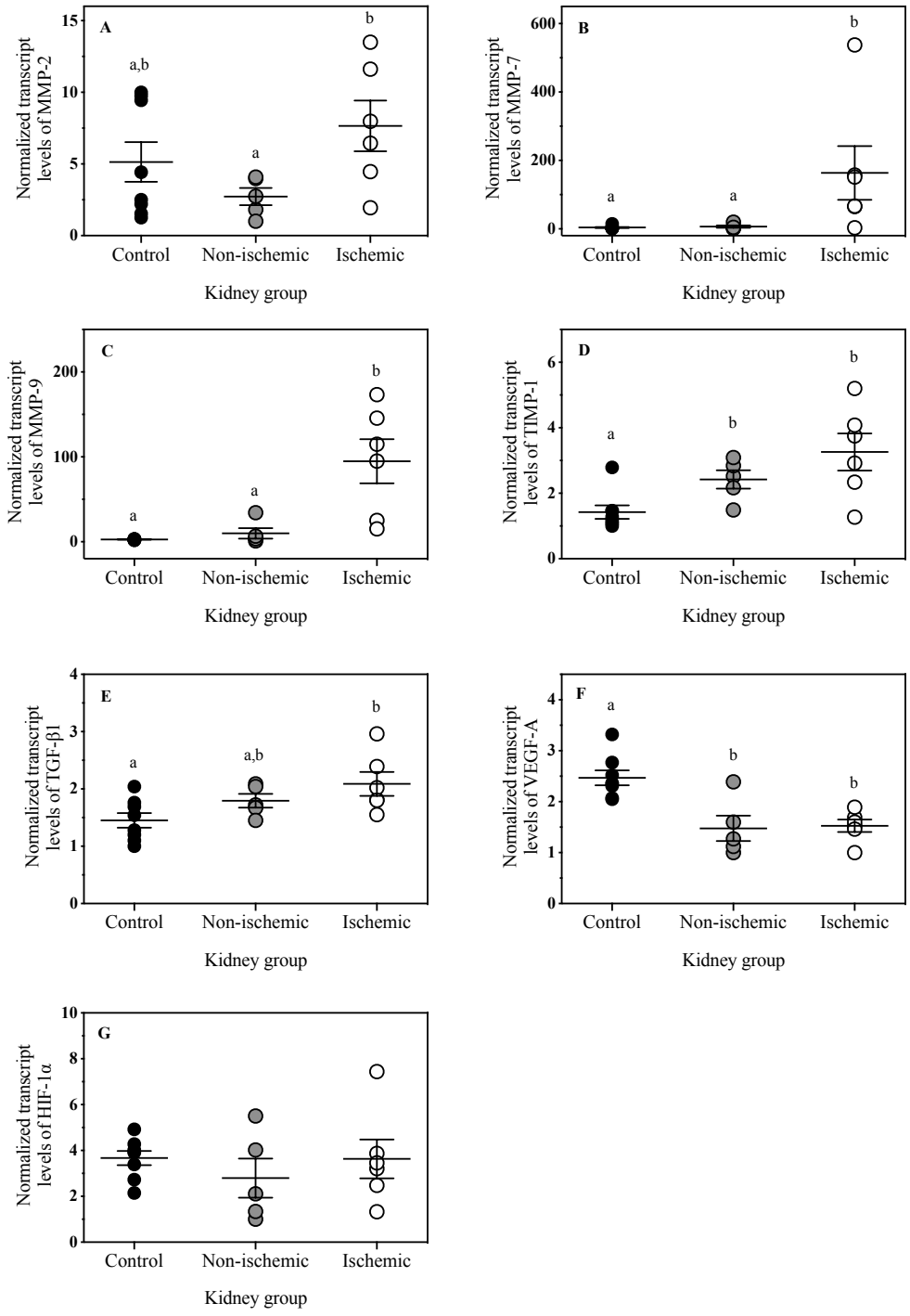

Figure 4.2. Dot plots of transcript levels of MMP-2 (A), MMP-7 (B), MMP-9 (C), TIMP-1 (D), TGF-β1 (E), VEGF-A (F) and HIF-1α (G) in renal tissue homogenates from normal control cats

(closed circles), and from the non-ischemic (gray circles) and ischemic kidneys (open circles) of cats that underwent 90 minutes of unilateral renal ischemia 6 months prior to euthanasia. Levels of each target gene were normalized to those of the reference genes GAPDH, RPS7 and ABL. Horizontal and error bars represent the mean and SEM levels for each kidney group. For each gene, transcript levels are scaled to that of the lowest sample; notice that scales differ among genes. [a,b] Groups with different letters differ significantly ($P < 0.05$).

Two genes were differentially expressed in both ischemic and contralateral kidneys from the RI cats versus control tissues: VEGF-A was bilaterally downregulated ($P = 0.002$) and TIMP-1 was bilaterally upregulated ($P = 0.011$) in the RI group kidneys. No differences in transcript levels of HIF-1α were observed ($P = 0.372$).

Histologic scores. Tissues from ischemic kidneys of the RI group had tubulointerstitial lesions characterized by tubular atrophy, and interstitial inflammation and fibrosis, as well as variable numbers of obsolescent glomeruli, consistent with the development of atubular glomeruli and subsequent ischemic glomerulosclerosis.[200] Consistent with study inclusion criteria, all control cats had normal renal histology with scores of 0 for fibrosis, inflammation, and tubular atrophy. Among samples from ischemic kidneys, median (range) fibrosis, inflammation, and tubular atrophy scores were 1.025 (0.3-3), 0.3 (0.2-1.65), and 1.025 (0.45-1.4), respectively (Figure 2). Conversely, all contralateral kidneys had histologic scores of 0, with the exception of one sample, for which the inflammation score was 0.05.

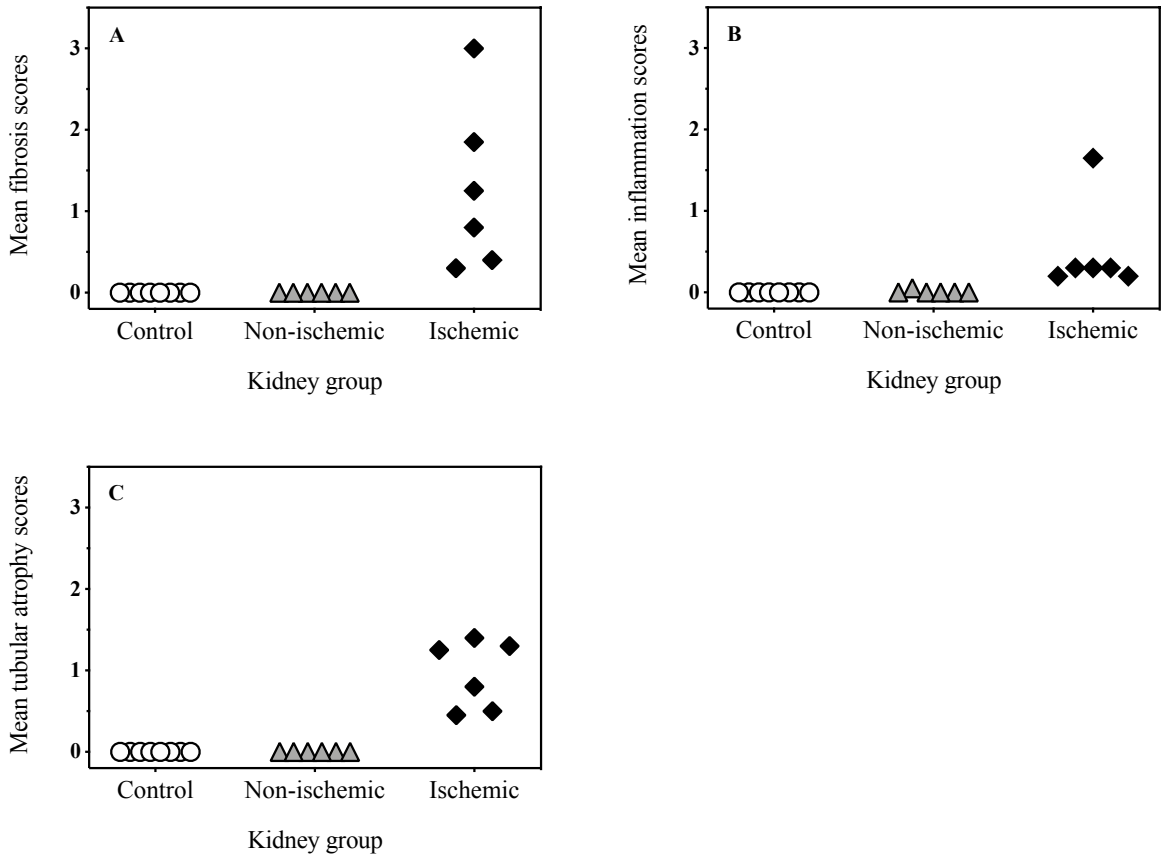

Figure 4.3. Dot plot of mean fibrosis (A), inflammation (B), and tubular atrophy (C) scores of kidneys from normal control cats (open circles), and of the non-ischemic (gray triangles) and ischemic kidneys (black diamonds) from cats that underwent 90 minutes of unilateral renal ischemia 6 months prior to euthanasia.

Correlation between transcript levels and histologic scores. Although no significant differences in transcript levels of HIF-1α were found among the ischemic and contralateral kidneys from the RI cats, and control kidneys from healthy cats, levels of HIF-1α were significantly and positively correlated with mean fibrosis score of the ischemic kidneys. Similarly, there was a significant positive correlation between the transcript abundance of MMP-2, MMP-7 and TIMP-1 and fibrosis of the ischemic kidneys (Table 2). There was no significant

correlation between transcription of any examined gene and mean inflammatory or tubular atrophy scores.

Table 4.4. Spearman rank correlation coefficients (r_s) quantifying the association between transcript levels of each gene and histologic scores for the ischemic kidneys (n = 6).

	Histologic scores		
Gene	Fibrosis	Inflammation	Tubular atrophy
HIF-1α	0.94**	0.00	0.60
MMP-2	0.94**	-0.28	0.66
MMP-7	0.88*	0.00	0.43
MMP-9	0.77	0.31	-0.03
TIMP-1	0.94**	0.15	0.43
TGF-β1	0.60	0.15	0.09
VEGF-A	-0.14	-0.62	0.43

* $P < 0.05$; ** $P < 0.01$

Discussion

Results of the present study show significant differences in the gene transcription of hypoxia-induced mediators of fibrosis in feline tissues collected six months after a single episode of transient, experimentally-induced renal ischemia. Overall, there was upregulation of MMP, TIMP-1 and TGF-β1 transcription, and downregulation of VEGF transcription, in renal tissues from the ischemia-induced CKD model, when compared to tissues from healthy controls. Additionally, there was a significant, positive association between the transcript abundance of specific mediators of fibrosis (i.e., HIF-1α, MMP-2, MMP-7 and TIMP-1) and the severity of the histologic lesions in ischemic kidneys, further supporting the role of these hypoxia-induced pathways in the development of fibrosis in CKD.

In the present study, altered transcription of genes associated with pro-fibrotic pathways was detected in tissues collected six months after transient renal insult, suggesting that a single such event can trigger a pro-fibrotic cascade that remains ongoing for months after the initial insult. Acute kidney injury and CKD, once seen as separate disease entities, are now recognized as interconnected syndromes.[188, 191] The results of this study support the link between acute kidney injury and CKD and identify specific cytokines that may be implicated in the progression of feline CKD. Further, because of its histological similarities to the advanced stages of human CKD,[24, 266] several forms of CKD of unknown etiology,[267, 268] and various tubulointerstitial nephropathies,[269, 270] feline CKD has been proposed as a naturally occurring model of the pro-fibrotic mechanisms driving human CKD progression.[203] Therefore, the results here presented may offer valuable information for the design of future translational research.

Hypoxia is a potent regulator of gene expression, with a broad range of molecular targets.[254] In CKD, renal hypoxia may be caused by decreased peritubular capillary blood flow due to imbalance in vasoactive factors, loss of capillary integrity, increase in oxygen demand from compensatory hyperfiltration and tubular hypertrophy, and increased oxygen diffusion distance between peritubular capillaries and tubular and interstitial cells due to accumulating ECM.[80-82] The resulting decrease in local oxygen tension worsens tubular injury, with tubular cell necrosis and tubular rupture, activation of resident interstitial fibroblasts, myofibroblast differentiation, accumulation of ECM, and recruitment of inflammatory cells culminating in tubulointerstitial inflammation and fibrosis, and further local hypoxia.[24, 32] Therefore, we hypothesized that pro-fibrotic pathways triggered by hypoxia would be differentially regulated in ischemia-induced CKD.

While there was a significant positive correlation between HIF-1α transcript levels and interstitial fibrosis in ischemic kidneys, there was no significant difference in HIF-1α levels between ischemic, contralateral and control kidneys. Hypoxia inducible factor-1 is a master regulator of oxygen homeostasis, playing critical roles in both cellular and systemic physiology and pathophysiology.[92] Increased renal HIF-1α expression is associated with tubulointerstitial injury in people with CKD.[96] Therefore, it was unexpected that transcription of this mediator was not similarly increased in the present study. Because tissues analyzed in this study were collected six months after ischemic injury, the role of this mediator may have been under-represented if HIF-1α expression is only transiently increased following an ischemic event. Further, the mechanisms by which hypoxia alters ECM metabolism in renal cells appear to involve both transcriptional and post-transcriptional events and occur via both HIF-1-dependent and -independent mechanisms,[254] and post-transcription regulation of expression was not evaluated by the present study. Importantly, although a difference in HIF-1α transcription among the different kidney groups was not observed, in ischemic kidneys, the transcript abundance of this gene was positively associated with the severity of fibrosis, suggesting that complex interactions *in vivo* may influence the relationship between gene transcription and resulting morphometric renal changes.

The MMPs are an important group of enzymes that regulate ECM composition.[98] Expression of most MMPs, normally low in tissues, is induced when remodeling of the ECM is required, and is primarily regulated at the transcriptional level.[98] Despite their critical role in matrix degradation, the MMPs are known to have both inhibitory and stimulatory roles in the regulation of fibrosis.[100, 102] Studies in rodent CKD models have documented the profibrotic effects of the collagenases MMP-2 and MMP-9,[77] and the matrilysin MMP-7,[102, 103] all of which

were upregulated in the present study. Upregulation of MMP-2 and -9 has also been documented in people with diabetic CKD.[271] Further, in children with CKD, serum concentrations of both MMP-2 and MMP-9, and their inhibitors TIMP-1 and TIMP-2, are elevated in proportion to disease stage.[107] MMP-7 also is recognized as both an important mediator of fibrosis in CKD, as well as a urinary biomarker of renal fibrosis.[103, 108] In people, urinary MMP-7 levels correlate with renal fibrosis scores.[272] Similarly, in our study, transcription of both MMP-2 and MMP-7 was significantly greater in ischemic kidneys compared to contralateral kidneys, and was significantly and positively associated with severity of interstitial fibrosis. These findings suggest that MMP-7 may prove to be a useful biomarker of renal fibrosis in cats, though studies evaluating the levels or activities of these proteins in cats with CKD are warranted.

Recent studies have exposed a close relationship between MMP-7 and TGF-β signaling in renal disease.[103] Activation of TGF-β triggers a series of events that promote fibrosis, including transcription of genes encoding matrix proteins, inhibitors of matrix-degrading enzymes and matrix-binding integrin receptors, transformation of fibroblasts into myofibroblasts, and chemotaxis of fibroblasts and monocytes.[112] In cats, urinary TGF-β1-to-creatinine ratio was increased in individuals with CKD when compared to healthy controls,[204] and in the present study, a significant positive correlation between transcription of TGF-β1 and severity of interstitial fibrosis was found.

In keeping with earlier findings of decreased urinary VEGF-to-creatinine ratio in cats with naturally occurring CKD,[205] results of the present study demonstrated decreased renal tissue transcription of VEGF-A. Loss of peritubular capillary density, possibly modulated by decreased VEGF or other angiogenic factors, has been proposed as a contributor to renal disease progression and aging-associated decline in renal function.[120] Cats with CKD have a pattern of

histologic change, even in early disease stages,[252, 253] that is characterized by marked tubulointerstitial change with limited glomerular injury. This pattern of change is corroborated in the model studied here and indicates that decreased VEGF-A may be a feature of certain types of CKD, although establishment of a causal relationship between a downregulation of VEGF-A and progression of renal disease requires further study. In fact, VEGF has been described as both protective and deleterious in different forms of CKD in people.[116] A distinction between VEGF and specifically VEGF-A has not been consistently made in these studies. Decreased urinary VEGF-to-creatinine ratio has been noted in cats with spontaneous CKD when compared to normal controls,[205] and this ratio was inversely correlated with the development of azotemia after treatment of hyperthyroidism in cats.[273] In a separate study however, increased urinary VEGF was associated with shorter survival and progression of azotemia in feline CKD.[a] Therefore, as is true for the other mediators evaluated here, the role of VEGF in the pathogenesis of CKD appears to be complex.

A relevant finding of the present study was the significant difference in transcript levels of VEGF-A (downregulated) and TIMP-1 (upregulated) in the contralateral, unmanipulated kidneys following transient unilateral ischemia, when compared to normal control kidneys. These findings suggest the existence of mechanisms for crosstalk between kidneys in response to unilateral injury. Potential mediators affecting gene transcription in contralateral kidney include circulating hormones, cytokines, or exosomes containing microRNAs,[274, 275] which were not assessed in the present study. Alterations to the contralateral kidney have also been observed in rodent models subjected to temporary unilateral renal ischemia. In rats, unilateral ischemia-reperfusion stimulated bilateral tumor necrosis factor-α production and neutrophil infiltration through a tumor necrosis factor-α-dependent mechanism.[276] Similarly, unilateral renal ischemia-

reperfusion induced inflammatory cell infiltration to both the ipsilateral and contralateral kidney, a finding that was present in aged, but not younger individuals.[277] Although inflammation was not present in the contralateral kidneys studied here, these cats were of a younger age. Whether unilateral injury might trigger both bilateral alterations to gene transcription and bilateral changes in renal inflammation remains to be examined in aged cats.

There are limitations to our study. First, study design was constrained by the availability of banked tissues from a relatively small number of subjects affected by an ischemic model of feline CKD.[200] Samples from a sham-operated control group were not available. Therefore, it is possible that the some of the alterations described, particularly those of the contralateral kidney, may have been caused by decreased renal perfusion during general anesthesia rather than by mechanisms of organ crosstalk following unilateral renal injury. Additionally, evaluation of the selected molecular patterns was performed exclusively by measurement of gene transcript levels. As evaluation of corresponding proteins was not performed, it is possible that post-transcription regulation results in protein levels that differ from what would be suggested by the present data. Animals in the RI group differed from controls in that they were older, had a greater mean body weight, and were ovariohysterectomized. It is therefore possible that the differences in age and neuter status may have influenced gene transcription results. Lastly, changes to renal transcription of pro-fibrotic pathways were evaluated at a single timepoint 180 days post-ischemia. Thus, early changes to renal gene transcription were not evaluated. This is of relevance as the observed histopathologic lesions (e.g., fibrosis) reflect chronic change, which would likely have been a result of alterations in gene expression at earlier time points – alterations that may or may not have been ongoing at the time of euthanasia and histologic evaluation. Because renal fibrosis contributes to chronic tissue hypoxia,[32, 255] the changes here reported may have been a

result of the observed tubulointerstitial fibrosis, rather than a cause for this pattern of histologic change. Future prospective studies may include a larger cohort, age- and gender-matched controls, and samples collected at earlier post-ischemia time points. Additionally, similar evaluation of naturally-occurring disease is necessary to draw conclusions regarding these molecular pathways in spontaneous disease.

In conclusion, differential regulation in transcription of major pathways of renal fibrosis was identified in an experimental model of feline CKD. Alterations of gene transcription in both kidneys were detected six months following transient unilateral ischemia. Future studies investigating these pathways in spontaneous CKD may identify new therapeutic targets and biomarkers of renal fibrosis in CKD.

Footnotes

a. Chakrabarti S, Syme HM, Elliott J. Urinary vascular endothelial growth factor as a prognostic marker in feline chronic kidney disease (abstract), *J Vet Intern Med* 2012;26:1524.

b. RNA*later* stabilization solution, QIAGEN, Valencia, CA.

c. RNeasy Plus Mini Kit, QIAGEN, Valencia, CA.

d. NanoDrop Spectrophotometer, Thermo Fisher Scientific, Waltham, MA.

e. Agilent 2100 Bioanalyzer system, Agilent Technologies, Palo Alto, CA.

f. ezDNase, Invitrogen, Carlsbad, CA.

g. SuperScript IV VILO Master Mix, Invitrogen, Carlsbad, CA.

h. SsoAdvanced SYBR Green Supermix, Bio-Rad Laboratories, Hercules, CA.

i. CFX96, Bio-Rad Laboratories, Hercules, CA.

j. PGEM-T easy vector, Promega, Madison, WI.

k. Molecular Cloning Laboratories, San Francisco, CA.

l. SPSS, IBM SPSS Statistics for Windows, version 24, IBM Corp., Armonk, NY.

m. GraphPad Prism for Mac, version 7, GraphPad Software Inc, La Jolla, CA.

CHAPTER 5

COMPREHENSIVE ANALYSIS OF PRO-INFLAMMATORY AND PRO-FIBROTIC PATHWAY UPREGULATION IN A FELINE MODEL OF ISCHEMIA-INDUCED CHRONIC KIDNEY DISEASE[3]

[3] Bianca N. Lourenço, Jaime L. Tarigo, DVM, Magdy S. Alabady, Amanda E. Coleman, Cathy A. Brown, Daniel R. Rissi, Scott A. Brown, and Chad W. Schmiedt. To be submitted to the American *Journal of Veterinary Research*.

Abstract:

Chronic kidney disease (CKD) is a common, progressive and irreversible disorder in domestic cats. As is the case for numerous types of human CKD, feline CKD is characterized by tubulointerstitial inflammation and fibrosis; however, the molecular pathways associated with its progression are incompletely understood. The objective of this study was to characterize the renal transcriptional activities in an ischemic model of feline chronic kidney disease using RNA sequencing. We hypothesized that 1) tissues from the CKD model would display increased genetic expression of molecular pathways associated with inflammation and fibrosis, and that 2) in cats having undergone unilateral renal ischemia, tissues from the contralateral, non-ischemic kidney would show differential genetic expression when compared to tissues from control cats.

Renal tissue samples banked from cats that underwent transient unilateral renal ischemia six months prior to tissue collection (n = 6) and from healthy, control cats (n = 9) were included in this study. For the experimental CKD cats, tissues from both the ischemic kidney (IK) and the contralateral, non-ischemic kidney (NIK) were evaluated. Illumina short read sequencing technology was used to produce renal transcriptome sequences using the RNAseq technique. Reads were mapped to the reference feline genome (Felis_catus_8.0). Differential expression analysis was conducted at a false discovery rate ≤0.05. Gene ontology and cluster analyses were subsequently performed.

Genes from the gene ontology categories of collagen binding (e.g., transforming growth factor β1), metalloendopeptidase activity (e.g., matrix metalloproteinase-7, -9, -11, -13, -16, -23B and -28), chemokine activity, and T-cell migration were overrepresented in the IK group vs. control. Genes associated with the extracellular matrix (e.g. tissue inhibitor of metalloproteinase-1 and connective tissue growth factor) were upregulated in both IK and NIK tissues.

Our results show that six months after transient renal ischemia, upregulation of inflammatory and pro-fibrotic pathways persists. Additionally, unilateral ischemic injury differentially alters gene expression in both kidneys.

Abbreviations

CKD, Chronic kidney disease

ECM, Extracellular matrix

FDR, False discovery rate

IK, Ischemic kidney

MMP, Matrix metalloproteinase

NGAL, Neutrophil gelatinase-associated lipocalin

NIK, Non-ischemic kidney

SPP1, Secreted phosphoprotein 1

TGF, Transforming growth factor

TIMP, Tissue inhibitor of metalloproteinase

TPM, Transcripts per million

VEGF, Vascular endothelial growth factor

Introduction

Chronic kidney disease (CKD) is a common disease in domestic cats.[182] Feline CKD shares common histologic features with end stage renal disease,[266] CKD of unknown etiology,[267, 268] and several tubulopathies in human beings,[269, 270] offering promise as a naturally occurring model for translational renal research.[203]

Our group has recently described two variations of a model of ischemia-induced CKD in cats, in which transient, unilateral renal ischemia induces chronic tubulointerstitial inflammation and fibrosis,[200, 225] changes that mimic those of naturally occurring disease in both the feline and human species.[183, 252, 253, 278] Using tissues from the later iteration of this model,[200] we reported upregulation of transcription of specific pro-fibrotic genes, including transforming growth factor (TGF)-β1, matrix metalloproteinases (MMP) -2, -7 and -9, and the tissue inhibitor of metalloproteinase (TIMP)-1 and downregulation of vascular endothelial growth factor (VEGF)-A that persisted 6 months following 90-minutes of unilateral renal ischemia in cats.[a] Importantly, unilateral renal ischemia induced changes in gene transcription of TIMP-1 and VEGF-A that affected both the previously ischemic kidney and the contralateral, undisturbed kidney of the experimental CKD cats. These findings support the role of renal hypoxia in the intrinsic progression of renal disease,[32, 254, 255] as well as the notion that acute kidney injury (AKI) and CKD are interconnected syndromes.[188, 191]

While the cat has been proposed as a naturally occurring model of CKD,[203] the molecular mechanisms of progression of feline CKD remain poorly understood. Comprehensive analysis of the renal transcriptome following renal ischemia may be crucial to identifying novel biomarkers and/or therapeutic targets for this highly prevalent disease.[279] The aim of this study was to characterize renal transcriptional activities in a feline model of ischemia-induced CKD using RNA sequencing. We hypothesized that 1) tissues from cats with experimental CKD would display increased genetic expression of molecular pathways associated with inflammation and fibrosis, and that 2) in cats having undergone unilateral renal ischemia, tissues from the contralateral, non-ischemic kidney would show differential genetic expression when compared to tissues from control cats.

Materials and Methods

Samples. This study included banked left and right kidney tissue samples from cats that underwent 90-minute unilateral renal ischemia six months prior to tissue collection (RI group, n = 6) and right kidney samples from healthy cats (control group, n = 9) that were evaluated in previously reported studies.[a,200] Tissues from both the ischemic kidney (IK group; right kidney) and the contralateral, non-ischemic kidney (NIK group; left kidney) were collected 6 months after renal ischemia. Banked tissue samples collected from healthy cats that were enrolled in unrelated terminal studies were also evaluated and used as controls.[a] Data reflecting renal function, including serum and urine biochemistry, and morphology were collected within 4 days of euthanasia.

Sample processing, RNA extraction and sequencing. Sample collection, processing and total RNA extraction was performed as previously reported.[a] Briefly, kidneys were collected via a midline laparotomy immediately after humane euthanasia. Each kidney was sectioned and one-fourth to one-half was minced and immediately placed in RNA*later* stabilization solution.[b] Following overnight incubation at 4°C, samples preserved in RNA*later* solution were decanted, individually homogenized using a mortar and pestle, divided into aliquots of 30 mg and stored at -80°C until analysis. Total RNA was extracted from one aliquot of renal homogenate per sample using a commercially available RNA extraction kit,[c] and quantified using a spectrophotometer.[d] Integrity of the isolated RNA was assessed first by visualization of 18S and 28S ribosomal bands on 1.2% agarose gels, followed by analysis using a bioanalyzer system.[e] Samples with a minimum RNA Integrity Number of 5.5 were used in the RNAseq analysis. Adequate RNA integrity was confirmed for all but one NIK sample; therefore, samples evaluated included six IK, five NIK, and nine control kidney samples. RNAseq library preparation and sequencing were

performed by the Georgia Genomics and Bioinformatics Core. Briefly, RNA samples were poly(A) enriched prior to cDNA synthesis to minimize the ribosomal RNA in the final libraries. The cDNA samples were converted to barcoded, stranded RNAseq libraries using the KAPA mRNA HyperPrep Kit,[f] according to the manufacturer instructions. The RNAseq libraries were assessed for concentration using the Kapa qPCR library quantification kit[g] and for size distribution using the High Sensitivity NGS Fragment Analysis kit[h]. All libraries were pooled using equi-molar amounts in a final RNAseq pool. The concentration and size distribution of the final pool were assessed prior to sequencing on the Illumina NextSeq 500 platform[i] using the paired-end 75 cycles sequencing kit.

Bioinformatic analyses. Sequencing data were demultiplexed based on the sample-barcodes map to assign reads to their samples using the Illumina bcl2fastq v2 pipeline. The RNAseq reads from each sample were quality-trimmed using Trimmomatic[280] to remove low-quality bases, homopolymers and artifacts. The Tuxedo Suite[281] was used to analyze the RNAseq data. First, quality reads were mapped to the reference feline genome (Felis_catus_8.0) using TopHat2. Second, Cufflinks was used to identify splice junctions and isoform detection and develop the per gene count matrix. Third, both edgeR[282] and DESeq2[283] algorithms were used to identify differentially expressed genes among the samples. To account for multiple testing errors, a false discovery rate (FDR) ≤ 0.05 was applied. In the differential expression analysis, we used the pairwise comparisons IK vs. NIK, IK vs. control, and NIK vs. control. The list of differentially expressed genes resulting from the pairwise comparisons were used for gene ontology analysis using the Panther classification system[284] and the Fisher's exact test with FDR multiple test correction.

Co-expression clustering analysis. We compiled a list of significantly expressed genes by taking the union of all significant genes from all comparisons in both edgeR and DESeq2 analyses (total = 4801 genes). Raw read counts were obtained from the RNAseq mapping data and were normalized using library size and gene length count to obtain the transcripts per million (TPM) scaled matrix. Using the Coseq algorithm,[285] we performed cluster analysis on the TPM matrix using different transformation and cluster models, and the following parameters were selected: cluster method = kmeans; cluster range to test = 2 to 25; transformation model = logCLR; minimum cuttoff = 5; number of starts = 1000; iteration max = 10000. The selected number of clusters was 20, which explained the majority of the variances among samples.

Results

Animals. Demographic and clinical characteristics of the cats from which the banked renal tissues originated are listed in Table 5.1. Cats that had undergone unilateral renal ischemia were older ($P = 0.002$) and heavier ($P = 0.006$) than control cats. These cats had been ovariohysterectomized at the time of surgically-induced renal ischemia, whereas control cats were intact. Despite the observed overlap in the available information on surrogate markers of renal function (i.e., serum creatinine and urea nitrogen concentrations, urine specific gravity and urinary protein-to-creatinine ratio), cats of the RI group were documented to suffer from a progressive decline in glomerular filtration rate and increase in serum creatinine concentration over the course of the original 6-month study.[200] Additionally, IK of the RI cats displayed lesions mimicking those of naturally occurring CKD, such as tubulointerstitial inflammation and fibrosis, whereas control cats had normal renal histopathology.

Table 5.1. Clinical and laboratory data from cats who underwent renal ischemia as a model of chronic kidney disease six months prior (n = 6) and from healthy control cats (n = 9). Numerical data are presented as median (range).

	Renal ischemia group*	**Control group**	**Laboratory reference interval**
Number	6	8	
Age (days)	520 (472-588)	270 (228-294)	
Sex	Female spayed	Female intact	
Body weight (kg)	4.72 (3.93-5.35)	3.59 (2.29-4.47)	
Serum creatinine concentration (mg/dL)	1.4 (1.3-1.6)	1.0 (0.8-1.2)	0.6-1.8
Serum urea nitrogen concentration (mg/dL)	24 (22-27)	28 (23-34)	21 – 36
Urine specific gravity	1.056 (1.041-1.059)	1.055 (1.037-1.061†)	N/A
Urinary protein-to-creatinine ratio	0.1 (0.05-0.11)	0.14 (0.1-0.2)	< 0.5

*For cats of the renal ischemia group, values for biochemical variables represent the average of two measurements obtained within 4 days of euthanasia.

†Urine specific gravities with results described as > 1.060 were assigned a value of 1.061.

Differentially expressed genes. Pairwise comparisons between IK, NIK and control kidneys revealed a total of 4739 and 5529 differentially expressed genes using edgeR and DESeq2, respectively.

In the data set obtained using edgeR, when tested against the control kidneys, the IK exhibited 1743 upregulated and 1196 downregulated genes, and the NIK displayed 209 upregulated and 291 downregulated genes. Compared to the NIK, there were 849 upregulated

genes and 451 downregulated genes in the IK. The genes previously known to be differentially transcribed among these three groups of kidneys (i.e., MMP-2, -7 and -9, TIMP-1, and VEGF-A)[a] were similarly represented in the present study.

Gene ontology analysis. Gene ontology analysis of the differentially expressed genes that were upregulated in the IK vs. control kidneys revealed an overrepresentation of several pro-inflammatory and pro-fibrotic pathways. Table 5.1 presents a summary of selected identified pathways according to their gene ontology category. When assessing these pathways based on their related biological processes, notable genes identified as significantly overrepresented and upregulated in IK included C-X-C motif chemokine-9, -10, -11 and -16 (T cell chemotaxis), T-cell-specific surface glycoproteins CD28 and CD5 (T cell costimulation), TGF-β1 and -β3, and vimentin (positive regulation of collagen biosynthetic process), CD74 (positive regulation of neutrophil chemotaxis), and platelet-derived growth factor receptor-α (platelet degranulation). Within the molecular function category, relevant genes identified in the pathways listed in table 5.2 included TGF-β1, -2 and -3, fibroblast growth factor-10 and -11, insulin-like growth factor-1, VEGF-C, hepatocyte growth factor and macrophage colony-stimulating factor 1 (growth factor activity), MMP-7, -9, -11, -13, -16, -23B and -28 (metalloendopeptidase activity), and connective tissue growth factor, fibrotectin, vascular cell adhesion protein-1, and intercellular adhesion molecule-1 (integrin binding). As for the cellular component category, pertinent genes included TIMP-1 and fibulin-1 (extracellular matrix).

Table 5.2. Select overrepresented pathways upregulated in previously ischemic kidneys (n = 6) of cats who underwent unilateral renal ischemia when compared to control kidneys (n = 9).

Gene ontology category	Overrepresented pathway	Fold enrichment	Raw *P*-value	FDR
Biological process	Dendritic cell chemotaxis (GO:0002407)	11.55	1.70E-03	3.49E-02
	T cell chemotaxis (GO:0010818)	10.83	1.39E-04	4.63E-03
	Positive regulation of macrophage chemotaxis (GO:0010759)	10.5	1.20E-05	5.59E-04
	Platelet degranulation (GO:0002576)	9.63	2.68E-03	4.91E-02
	T-helper 1 type immune response (GO:0042088)	9.02	9.38E-04	2.23E-02
	T cell migration (GO:0072678)	8.89	2.88E-05	1.19E-03
	T cell costimulation (GO:0031295)	8.02	1.38E-03	2.94E-02
	Positive regulation of collagen biosynthetic process (GO:0032967)	7.22	1.96E-03	3.86E-02
	Positive regulation of epithelial to mesenchymal transition (GO:0010718)	6.08	2.22E-04	6.81E-03
	Positive regulation of granulocyte chemotaxis (GO:0071624)	5.95	6.13E-04	1.58E-02
	Positive regulation of neutrophil chemotaxis (GO:0090023)	5.95	6.13E-04	1.57E-02
	Regulation of T cell proliferation (GO:0042129)	4.29	1.55E-07	1.13E-05
	T cell activation (GO:0042110)	4.28	3.87E-11	4.88E-09
	Positive regulation of angiogenesis (GO:0045766)	3.08	2.26E-04	6.87E-03
Molecular function	Toll-like receptor binding (GO:0035325)	9.63	2.20E-04	1.90E-02
	Collagen binding (GO:0005518)	6.8	5.28E-08	1.96E-05
	Chemokine binding (GO:0019956)	6.74	3.38E-04	0.0228
	Extracellular matrix binding (GO:0050840)	5.69	4.53E-06	0.0007
	Metalloendopeptidase activity	3.03	1.54E-04	9.78E-03
	Integrin binding (GO:0005178)	4.5	5.97E-07	0.00016
	Cytokine binding (GO:0019955)	3.61	4.57E-05	0.00514
	Heparin binding (GO:0008201)	3.51	2.14E-05	0.00274
	Growth factor activity (GO:0008083)	2.97	1.81E-05	0.00258
Cellular component	T cell receptor complex (GO:0042101)	9.63	2.20E-04	0.00665
	Protein complex involved in cell adhesion (GO:0098636)	7.67	5.02E-09	3.6E-07
	I complex (GO:0008305)	7.47	5.28E-08	3.3E-06
	MHC class II protein complex (GO:0042613)	7.22	6.89E-04	0.0185
	Extracellular matrix (GO:0005578)	3.38	5.09E-09	3.5E-07

Likewise, when comparing the IK and NIK of CKD model cats, similar pathways, represented by upregulated genes, were overrepresented in the former (Table 5.3).

Table 5.3. Select overrepresented pathways upregulated in previously ischemic kidneys (n = 6) when compared to non-ischemic kidneys (n = 5) of cats who underwent unilateral renal ischemia.

Gene ontology category	Overrepresented pathway	Fold enrichment	Raw *P*-value	FDR
Biological process	Establishment of T cell polarity (GO:0001768)	21.92	1.14E-03	4.04E-02
	Positive regulation of interleukin-2 biosynthetic process (GO:0045086)	16.7	3.26E-04	1.49E-02
	T cell costimulation (GO:0031295)	16.24	6.10E-05	3.82E-03
	Lymphocyte costimulation (GO:0031294)	16.24	6.10E-05	3.80E-03
	T cell chemotaxis (GO:0010818)	14.61	4.76E-04	2.03E-02
	Positive regulation of macrophage chemotaxis (GO:0010759)	10.63	1.21E-03	4.16E-02
	Positive regulation of neutrophil migration (GO:1902624)	10.31	8.00E-05	4.78E-03
	Positive regulation of granulocyte chemotaxis (GO:0071624)	10.31	8.00E-05	4.76E-03
	Positive regulation of interleukin-10 production (GO:0032733)	10.31	8.00E-05	4.74E-03
	Platelet activation (GO:0030168)	5.84	4.14E-04	1.83E-02
Molecular function	Cytokine receptor activity (GO:0004896)	6.94	5.23E-10	6.47E-07
Cellular component	T cell receptor complex (GO:0042101)	16.24	6.10E-05	3.17E-03
	MHC class II protein complex (GO:0042613)	14.61	1.69E-05	1.08E-03
	Integrin complex (GO:0008305)	6.05	9.18E-04	3.55E-02
	Extracellular matrix (GO:0031012)	3.17	2.26E-06	1.88E-04

While fewer genes were differentially expressed between the NIK and control kidneys, gene ontology analysis of genes upregulated in the NIK when compared with control kidneys

found that overrepresented pathways pertained, once again, to the regulation of inflammatory processes and extracellular matrix deposition (Table 5.4). Notable genes included in the overrepresented pathways included TIMP-1 and -2 (molecular function category of metalloendopeptidase inhibitor activity), as well as thrombospondin-1, MMP-28 and fibulin-1 (cellular component category of extracellular matrix).

Table 5.4. Select overrepresented pathways upregulated in non-ischemic kidneys (n = 5) of cats that underwent unilateral renal ischemia when compared to control kidneys (n = 9).

Gene ontology category	Overrepresented pathway	Fold enrichment	Raw P-value	FDR
Biological process	Regulation of chemotaxis (GO:0050920)	7.81	1.58E-04	4.20E-02
	Regulation of endopeptidase activity (GO:0052548)	5.52	1.31E-04	3.79E-02
Molecular function	Metalloendopeptidase inhibitor activity (GO:0008191)	63.04	3.49E-05	3.22E-02
	Cytoskeletal protein binding (GO:0008092)	3.71	1.65E-05	2.04E-02
Cellular component	Extracellular matrix (GO:0031012)	6.83	3.42E-07	1.42E-04

Gene co-expression clustering analysis. Using the selected criteria for gene co-expression clustering, 20 clusters explained the majority of the variances among samples (Figure 5.1).

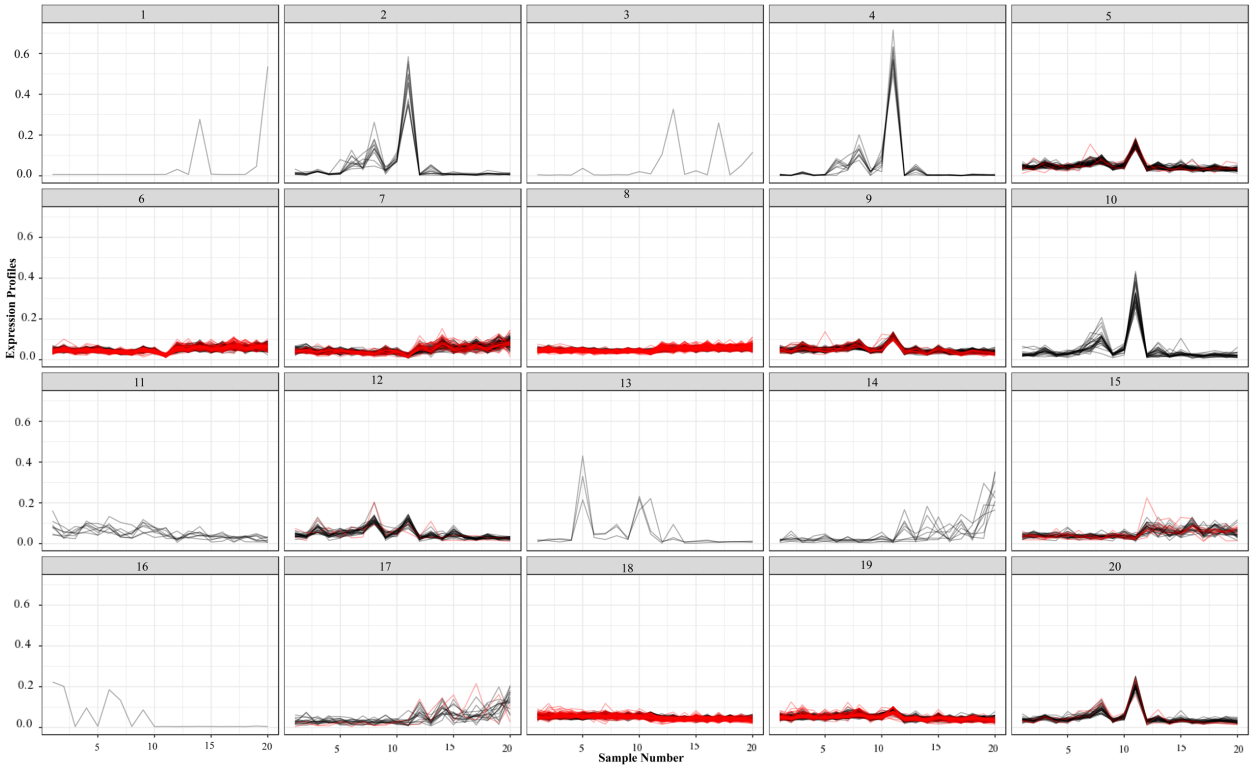

Figure 5.1. Profile plots for genes co-expressed in ischemic kidneys (n = 6) and non-ischemic kidneys (n = 5) from cats subjected to unilateral renal ischemia 6 months prior, and control kidneys obtained from healthy cats (n = 9). For each plot, the y-axis represents transformed, normalized gene expression and the x-axis represents individual renal samples. Samples 1-5 correspond to non-ischemic kidneys, and samples 6-11 to ischemic kidneys, of cats having undergone unilateral renal ischemia; samples 12-20 correspond to control kidneys. The red lines represent genes that have a maximum conditional probability of cluster membership (T_{max}) < 0.8), whereas black lines represent genes with $T_{max} \geq 0.8$.

Genes of interest, including secreted phosphoprotein 1 (SPP1) and matrix Gla protein, were identified in cluster 2 (Table 5.5) and cluster 10 (Table 5.6), respectively, displaying greater expression in IK, when compared to both the NIK and control kidneys.

Table 5.5. List of genes identified in cluster 2 as co-expressed in ischemic kidneys (n = 6), non-ischemic kidneys (n = 5) and control kidneys (n = 9).

Gene symbol	Description
SPP1	Secreted phosphoprotein 1
PLET1	Placenta expressed transcript 1
LOC109493266	Undetermined
LOC105261057	Undetermined
LOC105260308	Undetermined
LOC105260274	Undetermined
LOC105259930	Undetermined
LOC101098498	Predicted: Neutrophil gelatinase-associated lipocalin-like
LOC101090949	Undetermined
LOC101086711	Undetermined
JCHAIN	Joining chain of multimeric IgA and IgM

Table 5.6. List of genes identified in cluster 10 as co-expressed in ischemic kidneys (n = 6), non-ischemic kidneys (n = 5) and control kidneys (n = 9).

Gene symbol	Description	Gene symbol	Description
CCL21	C-C motif chemokine ligand 21	LOC101098301	DLA class II histocompatibility antigen, DR-1 beta chain
CD53	-	LOC102899262	Undetermined
CD74	-	LOC109494204	Undetermined
CFD	Complement factor D	LTF	Lactotransferrin
CHI3L1	Chitinase 3 like 1	MFAP4	Microfibril associated protein 4
CLDN3	Claudin 3	MGP	Matrix Gla protein
CLEC2D	C-type lectin domain family 2 member D	PI16	Peptidase inhibitor 16
CORO1A	Coronin 1A	PI3	Peptidase inhibitor 3
DRA	HLA class II histocompatibility antigen, DR alpha chain-like	PTPRC	Protein tyrosine phosphatase, receptor type C
IL7R	Interleukin 7 receptor	SLC26A3	Solute carrier family 26 member 3
IRF8	Interferon regulatory factor 8	SLC34A2	Solute carrier family 34 member 2
KRT7	Keratin 7	TACSTD2	Tumor associated calcium signal transducer 2
LCP1	Lymphocyte cytosolic protein 1		

Discussion

The results of the present study identify an extensive list of pro-inflammatory and pro-fibrotic pathways upregulated in the kidneys of cats previously subjected to transient, unilateral renal ischemia. Although six months elapsed between injury and sampling of renal tissues, thousands of genes involved in the regulation of inflammatory cell chemotaxis, cytokine and growth factor activities, and deposition of extracellular matrix were shown to be differentially expressed in pairwise comparisons of IK, NIK and control kidneys. Further, although direct ischemic injury was induced in only one of the kidneys of the RI group, pro-inflammatory and pro-fibrotic pathways were also upregulated in NIK when compared to control kidneys. Together with the results of a prior study that used reverse transcription-quantitative polymerase chain reaction to evaluate specific hypoxia-induced mediators of fibrosis transcribed in the same tissues, these data support AKI and CKD as closely related syndromes.[188, 191] Further, the present study, identifies additional cytokines that may be implicated in the progression of feline CKD.

Fibulin-1, an extracellular matrix protein prominently expressed in blood vessels,[286] was upregulated in the IK of the RI cats when compared to kidneys from healthy controls in the present study. In human beings, increased plasma fibulin-1 levels have been detected in individuals with various renal disease types, including glomerulonephritis, diabetic nephropathy, obstructive uropathy and analgesic abuse, therefore, this protein has been suggested as a marker for renal impairment.[287] In a separate report, plasma concentrations of fibulin-1 were inversely correlated with glomerular filtration rate, and positively correlated with systemic blood pressure and other hemodynamic markers of cardiovascular risk in CKD and diabetes.[288] Investigation of fibulin-1 plasma levels in cats with renal disease is warranted to assess this protein's potential as a biomarker for renal disease in this species.

In the present study, SPP1, also known as osteopontin, was upregulated in both kidneys from the RI group when compared to control kidneys. In rodent models and in people, this extracellular matrix protein and pro-inflammatory cytokine is highly expressed in renal tubular epithelial cells both *in vivo* and *in vitro*, and has been suggested as an important factor in the initial inflammatory responses involving NK cell activity in renal ischemia reperfusion injury.[289] In experimental rodent models of glomerulonephritis, expression of osteopontin correlates with the severity of associated tubulointerstitial injury.[290] Data from people and rodent models suggest that SPP1/osteopontin also plays a role in the pathogenesis of diabetic nephropathy.[291] In diabetic human beings, plasma osteopontin was independently associated with the presence and severity of nephropathy and coronary heart disease.[292] While data regarding osteopontin in feline CKD is lacking, gene co-expression clustering analysis here described revealed that SPP1/osteopontin is co-expressed with a predicted locus for neutrophil gelatinase-associated lipocalin (NGAL). As urinary NGAL has gained attention as a marker for the prediction of progression of feline CKD progression,[293] SPP1 may similarly offer promise as a biomarker for progressive CKD in cats.

Matrix Gla protein, an important vitamin K-dependent local inhibitor of vascular calcification,[294] was also upregulated in both IK and NIK when compared with control kidneys. Circulating levels of an inactive form of this matrix protein were positively and independently associated with aortic calcification in people with various stages of CKD, and proposed as a biomarker of vascular calcification in renal disease.[295] Additionally, in human renal biopsy samples from the Nephrotic Syndrome Study Network cohort, expression of matrix Gla protein was correlated with interstitial fibrosis, tubular atrophy, acute tubular injury, and interstitial inflammation, and positively associated with an increased risk of end-stage renal disease.[296] In our study, matrix Gla protein was co-expressed with several genes encoding for proteins

involved in inflammatory processes, such as lactotransferrin, a mediator of immune response to injury,[297] interleukin 7 receptor, and interferon regulatory factor 8, warranting further investigation of matrix Gla protein's potential as a marker for ongoing renal inflammation and fibrosis in cats.

There are limitations to this study. First, as banked tissues were available from only a small number of subjects affected by experimentally-induced CKD,[200] no sham-operated control group was available and the number of biological replicates in each group is relatively small. Second, animals in the RI group were older than control individuals, and were ovariohysterectomized. Such differences in age and reproductive status may have influenced gene transcription results; however, it appears unlikely that the transcriptional regulation of renal inflammation and fibrosis would have been impacted by these factors, particularly given that upregulation of several of the identified pro-inflammatory and pro-fibrotic pathways was noted when the IK were compared with the NIK of the same group of cats (i.e., the RI group). Finally, because tissues were collected at a single, relatively late, timepoint post-ischemia, earlier, transient players in the renal inflammatory processes may not have been detected. Future prospective studies may include a larger cohort, age- and gender-matched controls, and samples collected at multiple and earlier time points following renal ischemia.

Collectively, the data here reported suggest that acute, transient ischemic renal injury leads to persisting and wide-ranging changes in gene transcription that favor inflammation and fibrosis. Fibulin-1, secreted phosphoprotein-1 and matrix Gla protein may, as in human beings, prove to be useful biomarkers of active kidney injury in cats. Future evaluation of these proteins in renal tissues and/or plasma samples from cats with spontaneous CKD is warranted to explore their potential as renal biomarkers in this species.

Footnotes:

a. Lourenço BN, Coleman AE, Schmiedt CW, et al. Characterization of hypoxia-induced, profibrotic pathways in an ischemic model of feline chronic kidney disease (abstract), in *J Vet Intern Med*. 2018;32:2278.

b. RNA*later* stabilization solution, QIAGEN, Valencia, CA.

c. RNeasy Plus Mini Kit, QIAGEN, Valencia, CA.

d. NanoDrop Spectrophotometer, Thermo Fisher Scientific, Waltham, MA.

e. Agilent 2100 Bioanalyzer system, Agilent Technologies, Palo Alto, CA.

f. KAPA mRNA HyperPrep Kit, Kapa biosystems, Woburn, MA.

g. Kapa qPCR library quantification kit, Kapa biosystems, Woburn, MA.

h. High Sensitivity NGS Fragemnt Analysis kit, Advanced Analytical Technologies, Ankeny, IA.

i. Illumina NextSeq 500 platform, Illumina Inc., San Diego, CA

CHAPTER 6

EVALUATION OF PRO-FIBROTIC GENE TRANSCRIPTION IN NATURALLY-OCCURRING FELINE CHRONIC KIDNEY DISEASE[4]

[4] Bianca N. Lourenço, Amanda E. Coleman, Jaime L. Tarigo, DVM, Cathy A. Brown, Daniel R. Rissi, Scott A. Brown, and Chad W. Schmiedt. To be submitted to the *Journal of Veterinary Internal Medicine*.

Abstract

Background: Increased renal genetic transcription of hypoxia-induced mediators of fibrosis has been previously identified in an experimental model of feline chronic kidney disease (CKD).

Objective: To characterize hypoxia-induced, pro-fibrotic pathways in naturally-occurring feline CKD.

Animals: Thirteen client-owned cats, previously diagnosed with CKD and presented for humane euthanasia, and eight healthy control cats.

Methods: Bilateral renal tissue samples were assessed histologically for fibrosis, inflammation, and tubular atrophy, and by reverse transcription-quantitative polymerase chain reaction to characterize the transcript levels of hypoxia-inducible factor (HIF)-1α, matrix metalloproteinases (MMP)-2, -7 and -9, tissue inhibitor of metalloproteinase (TIMP)-1, transforming growth factor (TGF)-β1, and vascular endothelial growth factor (VEGF)-A. Gene transcription was compared among kidney groups and between kidneys of the same individual. Correlation between gene transcript levels and histologic scores for each kidney was also investigated.

Results: Kidneys from cats with CKD had significantly higher transcript levels of HIF-1α, MMP-2, -7 and -9, TIMP-1 and TGF-β1, and lower levels of VEGF-A than control cats. These differences remained after adjustment for cat age. Transcript levels of HIF-1α, MMP2, MMP-7 and TGF-β1 were positively correlated with worse histologic lesion scores in the CKD group.

Conclusion and clinical significance: Alterations in the renal transcription of genes involved in pro-fibrotic pathways were noted in cats with CKD. HIF-1α, MMP2, MMP-7 and TGF-β1 warrant investigation as potential biomarkers of renal fibrosis in cats.

Abbreviations

CKD, Chronic kidney disease

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

HIF, Hypoxia-inducible factor

IRIS, International Renal Interest Society

MMP, Matrix metalloproteinase

NCBI, National Center for Biotechnology Information

RPS7, Ribosomal protein S7

sCr, Serum creatinine concentration

SDMA, Serum symmetric dimethylarginine concentration

SUN, Serum urea nitrogen concentration

TGF, Transforming growth factor

TIMP, Tissue inhibitor of metalloproteinase

UPC, Urinary protein-to-creatinine ratio

USG, Urine specific gravity

VEGF, Vascular endothelial growth factor

Introduction

Tubulointerstitial fibrosis, a key histologic feature of feline chronic kidney disease (CKD),[252] is strongly correlated with the degree of functional renal impairment.[185, 253] This pattern of histologic change is commonly observed in the advanced stages of CKD in non-feline species, particularly as it progresses to end-stage renal disease.[24, 298] However, in cats, renal fibrosis is detected from the early disease stages.[252, 253] Although the processes eventually

leading to fibrosis may initially be part of normal tissue repair in response to renal injury,[299] fibrosis itself is proposed to eventually become a maladaptive response by altering the renal microcirculation and by increasing the distance for oxygen diffusion between renal tubules and capillaries, thereby promoting chronic renal hypoxia.[75, 198]

Accumulating evidence has emphasized the critical role of tubulointerstitial hypoxia in the development and progression of fibrosis and CKD.[198, 300, 301] Our group recently described increased transcription of known mediators of fibrosis, including the matrix metalloproteinases (MMP)-2, -7 and -9, tissue inhibitor of metalloproteinase (TIMP)-1 and transforming growth factor (TGF)-β1[a] in the kidneys of cats subjected to unilateral, transient renal ischemia six months prior to euthanasia as a model of feline CKD.[200] In that study, transcript levels of hypoxia-inducible factor (HIF)-1α, MMP-2, MMP-7, and TIMP-1 were positively correlated with increasing degrees of fibrosis in the previously ischemic kidneys. Additionally, changes in the gene transcript levels of two genes, TIMP-1 and vascular endothelial growth factor (VEGF)-A, were observed in not only kidneys directly subjected to transient ischemia, but also in the contralateral, unmanipulated kidneys, with VEGF-A bilaterally downregulated. In a separate study, analysis of the whole renal transcriptome in these same tissues showed differential regulation of thousands of genes when compared to normal controls. Interestingly, the same study identified approximately 500 genes that were differentially transcribed in the contralateral, non-ischemic kidney of the CKD model cats when compared to the tissues from healthy controls.[b] Collectively, these data illustrate that a single, transient hypoxic event may have lasting effects on the renal transcriptome, and that unilateral renal injury may induce changes to gene transcription in the contralateral kidney. Further, these studies recognize that specific

molecular pathways associated with renal fibrosis in other species appear to play a similar role in the early development of fibrosis in cats.

The objective of the present study was to characterize renal expression of hypoxia-induced, pro-fibrotic pathways in naturally-occurring feline chronic kidney disease. We hypothesized that gene transcript levels of HIF-1α, MMP-2, -7 and -9, TIMP-1 and TGF-β1 would be increased, and VEGF-A would be decreased in renal tissues from cats affected by CKD. A secondary objective was to determine whether significant differences in transcription of these genes are observed between kidneys of the same individual in cats with CKD, for which differences in gross morphology are often observed. We hypothesized that a greater difference in gene transcript levels would be observed between the left and right kidneys of individual cats with CKD, when compared to the differences noted between the left and right kidneys of individual healthy control cats.

Materials and methods

Study design. This was a prospective, cross-sectional study performed on renal tissue samples obtained from client-owned cats diagnosed with spontaneous CKD (CKD group) and from healthy control cats (control group).

The University of Georgia Institutional Animal Care and Use Committee approved all activities related to this study.

Animals. Tissue samples for the CKD group were obtained postmortem from cats presented for their final visit at primary care and referral veterinary hospitals in the states of Georgia and North Carolina. Cats were considered for enrollment if they were documented or suspected to be affected by CKD, based on historical fulfillment of at least one of the following

criteria: urine specific gravity (USG) < 1.035 with no identifiable extra-renal cause, azotemia (serum creatinine concentration [sCr] ≥ 1.6 mg/dL), serum symmetric dimethylarginine concentration (SDMA) > 14 μg/dL, and renal proteinuria (urinary protein-to-creatinine ratio [UPC] > 0.4). Cats were included if renal histopathologic analysis, performed by one of two veterinary pathologist (CAB or DRR), revealed chronic lesions (i.e., glomerulosclerosis, tubular atrophy, and/or tubulointerstitial fibrosis). Cats could be humanely euthanized or die of natural causes due to either renal or extra-renal disease. Cats were excluded if they had received a renin-angiotensin-aldosterone system antagonist (i.e., an angiotensin-converting enzyme inhibitor, angiotensin receptor blocker, or mineralocorticoid receptor antagonist) or a corticosteroid at any point in the 14 days preceding euthanasia. Cats were also excluded if they were affected by uncontrolled hyperthyroidism (Total T4 > upper limit of laboratory reference range at last sampling) or congestive heart failure. All owners were required to read and sign a form consenting to their pets' participation in the study.

Samples from the control group were collected from adult cats that were humanely euthanized as part of population control measures at a local animal control facility, and from adult purpose-bred cats participating in unrelated terminal studies with no impact on renal structure and function. These cats were considered to be structurally healthy based on normal findings of necropsy and renal histopathology, and were deemed to have normal renal function on the basis of serum biochemistry and urine analyses (i.e., sCr <1.6 mg/dL, SDMA ≤ 14 μg/dL, USG > 1.035, and UPC ≤ 0.4). For healthy intact male cats, a UPC < 0.6 was considered acceptable for inclusion, provided all other renal function biomarkers (i.e., sCr, serum urea nitrogen [SUN], SDMA and USG) and renal histopathology were normal.[302] Cats recruited at the animal control facility were additionally screened for retroviral infections using a combo feline

immunodeficiency virus antibody and feline leukemia virus antigen test,[c] and were excluded if positive for either.

Blood and urine sample collection and processing. For control cats identified at the animal control facility, blood and urine samples were collected immediately following euthanasia via cardiocentesis and cystocentesis, respectively. For purpose-bred control cats, blood and urine samples were collected via jugular venipuncture and cystocentesis while under sedation (buprenorphine 0.03 mg/kg IM, acepromazine 0.1 mg/kg IM and midazolam 0.3 mg/kg) and immediately prior to euthanasia with pentobarbital. Immediately following collection, blood samples were placed in anti-coagulant free and EDTA-containing glass tubes, and urine was placed in glass tubes. Anti-coagulated blood and urine samples were transported on ice. Anti-coagulant free samples were transported at room temperature, allowing clot formation. All samples were delivered to the Clinical Pathology Laboratory the University of Georgia College of Veterinary Medicine for analyses within one hour of collection.

Client-owned cats enrolled in the CKD group did not undergo blood and urine sampling. Information pertaining to their renal function was obtained upon review of the individual's medical record.

Clinical laboratory analyses. Complete blood count, serum biochemistry profile, serum SDMA measurement, urinalysis and UPC measurement were performed for all control cats. Serum SDMA was measured by an external laboratory.[d] All other clinical laboratory analyses were performed by the Clinical Pathology Laboratory of the University of Georgia.

Renal tissue sample collection and processing. For all cats, renal tissue samples were collected within one hour of death or euthanasia by one of the investigators or a trained individual. For each cat, both kidneys were removed through a midline laparotomy and sectioned

longitudinally. To maintain equal proportions of renal cortex, medulla and corticomedullary junction, one-half of each kidney was minced and placed in RNA stabilization solution,[e] and the remaining portion was placed in neutral-buffered 10% formalin. Following overnight incubation at 4°C, tissues were removed from RNA stabilization solution, homogenized with a mortar and pestle, divided into 30 mg aliquots, and stored at -80°C until further analysis.

Gene transcription analysis. Reverse transcription and quantitative polymerase chain reaction were performed as previously described.[a] For each sample, total RNA was extracted from 30 mg of tissue homogenate using a commercially available RNA extraction kit.[f] The integrity of the isolated RNA was confirmed by quantification using a spectrophotometer,[g] followed by the visualization of 18S and 28S ribosomal bands on 1.2% agarose gels. A total of 1 μg of RNA extracted from each sample was treated with deoxyribonuclease,[h] and reverse-transcribed using a cDNA reaction master mix.[i] Quantification was performed in an automated cycler[j] using 20 μL reactions containing 10 μL of SYBR Green Supermix,[k] 5 pmol of each primer (Table 6.1), and 9 μL of complementary DNA sample at a 1:40 dilution. Thermal cycling conditions consisted of an activation step at 95°C for 30 seconds, followed by 36 amplification cycles (95°C for 15 seconds for denaturation and 60°C for 30 seconds for annealing and extension), and a melt curve step (60°C to 95°C, increasing at increments of 0.5°C every 5 seconds). All reactions were performed in triplicate and average values were used for further analyses. A no reverse transcriptase control was included for each sample and three no template controls were used in each plate. In accordance with the sample maximization method,[259] for each gene, all samples were analyzed in two 96-well plates. A standard sample was analyzed in triplicate with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers in each plate and used as an inter-run control for the entire gene study.

Table 6.1. Primer sequences for quantitative polymerase chain reaction.

Gene	Ensemble ID/ NCBI Access. No.	Primer sequence (5'→ 3')	Fragment size (bp)	Source
GAPDH	ENSFCAG 00000006874	for: GCTGCCCAGAACATCATCC rev: GTCAGATCCACGACGGACAC	134	Riedel et al.[260]
RPS7	NM_001009832	for: GTCCCAGAAGCCGCACTT T rev: CACAATCTCGCTCGGGAA AA	74	Kessler et al. [261]
HIF-1α	XM_001493206	for: TTGGCAGCAATGACACAGACACTG rev: TTGAGTGCAGGGTCAGCACTACTT	175	Agaoglu et al.[262]
MMP-2	XM_003998042.2	for: AGACAAGTTCTGGAGGTACAATG rev: CGCCCTTGAAGAAGTAGCTGT	149	NCBI Primer-BLAST
MMP-7	XM_003992303.2	for: CTTTGCAAGAGGAGCTCACG rev: AATTCCTAGACCCCTGCCGT	148	NCBI Primer-BLAST
MMP-9	XM_003983412.4	for: GCTTCTGGAGGTTCGACGTG rev: CAATAGAAGCGGTCCTGGCA	148	NICBI Primer-BLAST
TGF-β1	AY425617.1	for: AGCACGTGGAGCTGTACCAGAAAT rev: TCCAGTGACATCAAAGGACAGCCA	110	Agaoglu et al.[263]
TIMP-1	XM_011291721.2	for: TCTCATCGCCGGAAAACTGC rev: AGCCAGCAGCATAGGTCTTG	122	NCBI Primer-BLAST
VEGF-A	AB071947.1	for: TTTCTGCTCTCTTGGGTGCATTGG rev: TGCGCTGGTAGACATCCATGAACT	139	Agaoglu et al.[262]

Transcript levels of the target genes HIF-1α, MMP-2, MMP-7, MMP-9, TGF-β1, TIMP-1 and VEGF-A were normalized to those of two reference genes, GAPDH and ribosomal protein S7 (RPS7), using the GENorm method.[264] Reference genes were selected based on the efficiency of their primers, and their stability value (M value < 0.5) and coefficient of variance (< 0.25) when tested in a complete set of the experiment's samples. After normalization, for each target gene, transcript levels were scaled to those of the lowest sample.

Gene specific primers were selected from previously reported studies, when available, or designed using the NCBI primer-BLAST[265] (Table 6.1). Primer efficiencies were calculated using the Pfaffl method based on standard curves performed with serial dilutions of cDNA (factor of two or four; three replicates) starting at a 1:20 dilution. Quantitative PCR products were confirmed on 1.2% agarose gels. Amplicons were cloned into a vector system[m] and

sequenced bi-directionally by chain termination.[l] Resulting sequences were confirmed via NCBI-BLAST against the feline genome.[265]

Histopathology. Three-micron thick sections of paraffin-embedded, formalin-fixed tissues were stained with Masson's trichrome, hematoxylin and eosin, and periodic acid-Schiff and hematoxylin. Ten consecutive 20x cortical fields were scored for the degree of fibrosis, inflammation, and tubular atrophy by two board-certified veterinary pathologists (both CAB and DRR), as previously described.[200] The grading scheme for each histologic score is presented in table 6.2. Numeric scores of the 10 examined fields were averaged to generate mean scores for inflammation, tubular atrophy, and fibrosis for each kidney.

Table 6.2. Renal histologic scoring system for inflammation, tubular atrophy and fibrosis.

	Histologic scores		
Score	Inflammation	Tubular atrophy	Fibrosis
0	No inflammatory cells	No atrophy	Absent, no increase compared to normal
1	Mild, < 10% of interstitium affected	Mild, fewer than 10 scattered atrophic tubules	Mild, rare foci/segments of fibrosis involving < 20% of cortex
2	Moderate, 20-50% of field affected	Moderate, linear streaks of tubular atrophy often with fibrosis and inflammation	Moderate, fibrotic segments involving 20 – 30% of cortex
3	Severe, > 50% affected	Severe, two or more streaks of tubular atrophy	Severe, fibrotic segments involving > 30% of cortex

Statistical analysis. Statistical analyses were performed using commercially available software packages.[n,o] A significance threshold of 0.05 was used.

The distribution of age, body weight, sCr, USG and UPC data by study group was examined for normality by visual assessment of histograms and normal quantile plot, and the Shapiro-Wilk test. Normally distributed data are presented as mean ± SD and compared between groups using the Student's t test. Non-normally distributed data are presented as median (range)

and compared using the Mann-Whitney U test. For purposes of comparison between groups, USG values reported as >1.060 and SUN values reported as >100 mg/dL were assigned a value of 1.061 and 101, respectively.

Histograms and Q-Q plots of scaled normalized gene transcript levels linear mixed model residuals were examined to evaluate the assumption of normality. Model residuals were right-skewed and unequal between groups and were therefore natural log transformed prior to analysis which improved the normality of the residuals and homogeneity of variances.

Linear mixed models were used to compare natural log-transformed, scaled, normalized gene transcript levels between groups. The full model for each gene expression variable included a fixed factor for group and random factor of cat to account for within-cat correlation. Alternative models also included a fixed covariate of age to adjust for effects of age or sCr and a sCr by group interaction to test for association of creatinine and gene transcription variables. A fixed covariate of histologic score and a histologic score by group interaction was used to test for association of each score and gene transcription variables. Estimate of Satterthwaite degrees of freedom method and restricted maximum likelihood estimation were used.

Absolute differences in natural log-transformed scaled normalized gene transcript levels between left and right kidneys were calculated. The folded form F statistic was used to test if variances in differences were equal between groups for each gene transcript level difference variable. If unequal, then the Welch-Satterthwaite t-test was used to compare absolute differences between groups.

Pearson correlations were used to test for correlations of sCr and histologic scores and natural log-transformed, scaled, normalized gene transcript levels for each gene variable and group separately. An analysis of variance with factors of group and histologic score was used to

test for associations of scores and natural log-transformed, scaled, normalized gene transcript levels for each gene variable in both groups together.

Results

Animals. Samples from 13 cats with a diagnosis of CKD and from 12 control cats (five purpose-bred and seven feral cats) were collected between July 2017 and March 2019. Tissues from one cat with CKD were collected by his primary care veterinarian in Morrisville, NC. For all other CKD and control cats, tissue collection was performed by the study investigators in Athens, GA or Atlanta, GA. Four control cats did not meet the enrollment criteria and their samples were, therefore, excluded from analysis. One purpose-bred cat was excluded due to unexplained, marked right hind limb edema. Reasons for exclusion of three feral cats included a concurrent diagnosis of pyometra (n=1), positive feline immunodeficiency virus antibody test (n = 1), and concurrent leukocytosis and bacteriuria (n = 1). Thirteen cats with CKD and eight healthy control cats met the enrollment criteria and were included in this study.

A summary of the demographic and clinical information of the cats included in this study is presented in table 6.3. While the age of four feral control cats was estimated, cats included in the CKD group were significantly older ($P < 0.001$) than the healthy controls. Cats of both genders were represented in each group; however, a greater proportion of sexually intact cats was included in the control group ($P = 0.01$). There was no significant difference in body weight or breed distribution between groups ($P = 0.25$ and $P = 0.21$, respectively).

Table 6.3. Clinical and laboratory data from cats with chronic kidney disease (n = 13) and from healthy control cats (n = 8). Numerical data are presented as mean ± SD or median (range), where appropriate. Number of cats for which data is available is provided if different from the number of individuals in each group.

	Chronic kidney disease group	**Healthy control group**
Number included	13	8
Age (years)	17.3 (3.1-19.4)	1.7 (1-7)
Sex		
Female spayed	7	0
Female intact	0	3
Male neutered	6	4
Male intact	0	1
Breed		
Domestic short haired	9	8
Domestic medium haired	3	0
Abyssinian	1	0
Body weight (kg)	3.85 ± 1.37	4.22 ± 0.95
n	11	8
Serum creatinine concentration (mg/dL)	4.8 ± 3.4	0.9 ± 0.5
Serum urea nitrogen concentration (mg/dL)	73.9 ± 33.7	24.9 ± 5.1
Serum symmetric dimethylarginine (µg/dL)	9.5 (9-20)	10.5 (6-14)
n	4	8
Urine specific gravity	1.018 (1.011-1.061)	1.048 (1.040-1.051)
n	9	8
Urinary protein-to-creatinine ratio	0.69 (0.39-23.41)	0.19 (0.07-0.41)
n	4	8
IRIS CKD stage*		
1	3	
2	2	N/A
3	2	
4	6	

* IRIS CKD stage assumes that the last recorded measurement represented the cat's stable value.

All cats in the CKD group were euthanized for clinical signs presumed to be related to kidney disease, including persistent lethargy, inappetence and chronic weight loss. Acute-on-chronic kidney disease was considered likely for two of these cats. The median (range) time between the date of last creatinine measurement and euthanasia for CKD cats was 6 (0-525) days. Assuming that each cat's last creatinine was stable, cats included in the CKD group would have been classified as International Renal Interest Society (IRIS) CKD stages 1 through 4, with a minimum of 2 cats in each stage. Systolic blood pressure and UPC measurements were not consistently available for CKD substaging.

Concurrent diseases/conditions were diagnosed in 6 cats from the CKD group and included hypertrophic cardiomyopathy (n = 1), pancreatic Langerhans cell histiocytosis, extensive necrotizing enteritis and mild myocardial hypertrophy (n = 1), possible Streptococcus sp. (alpha) urinary tract infection Streptococcus sp. (growth from subculture of enrichment broth – uncertain clinical relevance) undergoing antibiotic treatment for 13 days (n = 1), bilateral nephrolithiasis with left ureteral obstruction treated with ureterotomy 16 days prior (n = 1), unilateral nephrolithiasis (calcium oxalate), gastrointestinal and pancreatic lymphoma, thyroid adenoma, diabetes mellitus, and left ventricular concentric hypertrophy (n = 1), and suspect middle ear neoplasm (n = 1).

Medications being administered in the period prior to euthanasia included mirtazapine (n = 2), amoxicillin/clavulanic acid (n = 2), atenolol (n = 1), calcium carbonate (n = 1), aluminum hydroxide (n = 1), ranitidine (n = 1), sucralfate (n = 1), lactulose (n = 1) and NPH insulin (n = 1). Subcutaneous fluids were being administered regularly at home in two cases.

Seven cats in the CKD group were being fed a prescription renal diet (Hill's k/d in six cases and Royal Canin Feline Renal Support D in the remaining case). One cat was being fed a maintenance diet (Pro Plan Focus urinary tract formula). Dietary information was not available for five cats.

Gene transcription analysis. Renal tissue samples from the CKD group had significantly higher transcript levels of HIF-1α, MMP-2, MMP-7, MMP-9, TIMP-1 and TGF-β1 (all $P < 0.001$), and lower levels of VEGF-A ($P = 0.006$) than those of the control group (Figure 6.1). Age was not significantly associated with transcript levels of any of the seven genes evaluated (Table 6.4). Differences in transcript levels of all genes remained statistically significant when adjusted for the difference in age between the cats in each group (all $P < 0.03$; Table 6.5).

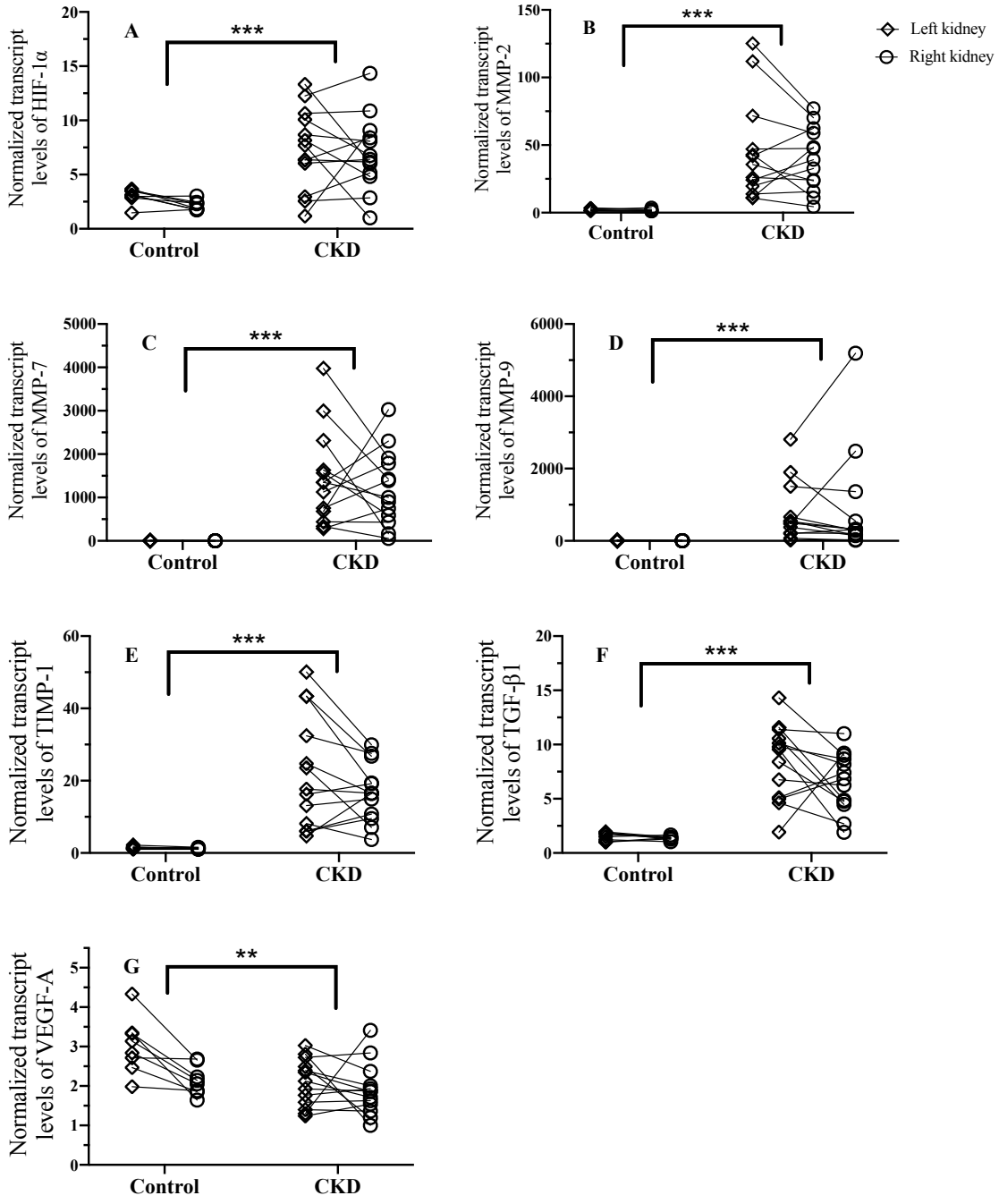

Figure 6.1. Dot plots of normalized gene transcript levels of HIF-1α (A), MMP-2 (B), MMP-7 (C), MMP-9 (D), TIMP-1 (E), TGF-β1 (F), and VEGF-A (G) in renal tissue homogenates from each kidney of cats with chronic kidney disease (n = 13) and healthy control cats (n = 8). Levels of each target gene were normalized to those of the reference genes GAPDH and RPS7. For each gene, transcript levels are scaled to those of the lowest sample; notice that scales differ among

genes. Values from the left (open diamond) and right kidney (open circle) of the same individual are connected by solid lines. ** *P*-value < 0.01; *** *P*-value <0.001

Table 6.4. *P*-values for the association between age and transcript levels of each gene.

Gene	P-value
HIF-1α	0.972
MMP-2	0.931
MMP-7	0.267
MMP-9	0.632
TGF-β1	0.976
TIMP-1	0.911
VEGF-A	0.588

Table 6.5. *P*-values for the comparison of gene transcript levels from cats with chronic kidney disease (n = 13) and from healthy controls (n = 8) using linear mixed models with (adjusted) and without (un-adjusted) including age as a covariate.

Gene	Un-adjusted *P*-value	Adjusted *P*-value
HIF-1α	<0.001	0.013
MMP-2	<0.001	<0.001
MMP-7	<0.001	<0.001
MMP-9	<0.001	<0.001
TGF-β1	<0.001	<0.001
TIMP-1	<0.001	<0.001
VEGF-A	0.006	0.034

The absolute differences in natural log-transformed, scaled, normalized gene transcript levels between kidneys of the same individual were significantly higher in CKD cats than in controls for TGF-β1 ($P = 0.040$) and TIMP-1 ($P = 0.004$). Descriptive statistics for these absolute differences are presented in table 6.6.

Table 6.6. Descriptive statistics of absolute differences between kidneys in un-transformed and ln-transformed scaled normalized gene transcript levels.

Gene	Group	N	Variable	Mean	Lower 95% CL for mean	Upper 95% CL for mean	Std Dev	Min	Max
HIF-1α	Control	8	diff.	0.96	0.45	1.47	0.61	0.06	1.81
			ln diff.	0.38	0.18	0.57	0.23	0.02	0.68
	CKD	13	diff.	2.81	1.13	4.5	2.79	0.22	7.9
			ln diff.	0.55	0.12	0.97	0.71	0.02	2.04
MMP-2	Control	8	diff.	0.69	0.26	1.12	0.52	0.2	1.41
			ln diff.	0.31	0.2	0.42	0.13	0.17	0.51
	CKD	13	diff.	18.7	9.01	28.39	16.03	0.55	48.06
			ln diff.	0.53	0.25	0.8	0.45	0.01	1.46
MMP-7	Control	8	diff.	2.16	0.82	3.5	1.6	0.34	4.82
			ln diff.	0.71	0.29	1.13	0.5	0.1	1.7
	CKD	13	diff.	1014.4	548.71	1480.03	770.58	8.88	2345.7
			ln diff.	0.91	0.49	1.33	0.69	0.02	2.63
MMP-9	Control	8	diff.	4.73	-1.49	10.96	7.44	0.06	20.11
			ln diff.	0.48	0.12	0.85	0.44	0.03	1.25
	CKD	13	diff.	575.79	90.61	1060.96	802.88	10.28	2388.8
			ln diff.	0.76	0.46	1.07	0.5	0.05	1.66
TGF-β1	Control	8	diff.	0.36	0.23	0.49	0.15	0.14	0.59
			ln diff.	0.25	0.18	0.33	0.09	0.09	0.38
	CKD	13	diff.	3.6	1.99	5.2	2.66	0.39	7.64
			ln diff.	0.59	0.27	0.9	0.52	0.03	1.61
TIMP-1	Control	8	diff.	0.21	0.03	0.4	0.22	0.02	0.59
			ln diff.	0.14	0.03	0.25	0.13	0.02	0.36
	CKD	13	diff.	9.28	4.64	13.92	7.68	1.15	24
			ln diff.	0.54	0.31	0.77	0.39	0.07	1.25
VEGF-A	Control	8	diff.	0.88	0.35	1.4	0.63	0.03	1.7
			ln diff.	0.33	0.14	0.52	0.23	0.01	0.71
	CKD	13	diff.	0.6	0.19	1.02	0.69	0.04	2.11
			ln diff.	0.31	0.09	0.53	0.36	0.03	1.03

Correlation between gene transcript levels and serum creatinine concentration. There was a significant, positive association of sCr and the transcript levels of MMP-2 in both control and CKD groups (when considering the Pearson's correlation coefficient), and between sCr and the transcript levels of each of MMP-7 and TIMP-1 in the CKD group (when considering both statistical approaches). Pearson's correlation coefficients (r) and respective *P*-values, and slopes (unit change in ln-transformed transcript levels per 1 mg/dL change in creatinine) and *P*-values from a linear mixed model testing these associations are presented in table 6.7.

Table 6.7. Tests of association of serum creatinine concentration and gene transcript levels using the Pearson's correlation coefficient or a linear mixed model.

Gene	Group	r[a]	*P*-value[a]	Slope[b]	*P* -value[b]
HIF-1α	Control	-0.35	0.183	-0.21	0.534
	CKD	0.04	0.838	0.01	0.824
MMP-2	Control	0.55	0.028	0.49	0.272
	CKD	0.46	0.018	0.12	0.037
MMP-7	Control	0.42	0.107	0.53	0.243
	CKD	0.47	0.016	0.15	0.015
MMP-9	Control	0.64	0.008	1.14	0.210
	CKD	0.24	0.231	0.11	0.315
TGF-β1	Control	0.45	0.084	0.19	0.428
	CKD	-0.05	0.804	-0.01	0.767
TIMP-1	Control	0.1	0.705	0.04	0.892
	CKD	0.58	0.002	0.13	0.003
VEGF-A	Control	-0.02	0.949	-0.01	0.953
	CKD	-0.23	0.248	-0.02	0.222

[a] Pearson's correlation

[b] Linear mixed model with fixed effect = group, creatinine, group*creatinine; random effect = cat

Gross and histologic evaluation of kidneys. Marked asymmetry in renal size and morphology was present in four CKD cats (i.e., "big kidney-little kidney" syndrome). In each of these cases, the right kidney was enlarged while the left kidney was small.

On histology, cats with CKD were diagnosed with typical ischemic CKD (i.e., chronic tubular atrophy and tubulorrhexis with interstitial inflammation, lipid, and fibrosis; n = 8), ischemic CKD with oxalosis (n = 2), amyloidosis (n = 1), membranoproliferalive glomerulonephritis (n =1), and possible renal maldevelopment with secondary ischemic changes (n = 1). Urothelial hyperplasia was noted in seven cats with ischemic CKD.

Histologic scores. In accordance with inclusion criteria, control cats had normal renal histologic evaluation with median (range) mean cortical scores of inflammation, tubular atrophy, and fibrosis of 0 (0-0.2), 0 (0-0.3), and 0 (0-0), respectively. For CKD cats, median (range) inflammation, tubular atrophy, and fibrosis scores were 1.7 (0.4-2.7), 1.1 (0.2-2.4), and 1.75 (0.3-3.0), respectively, with numeric differences noted between kidneys of the same cat (Figure 6.2).

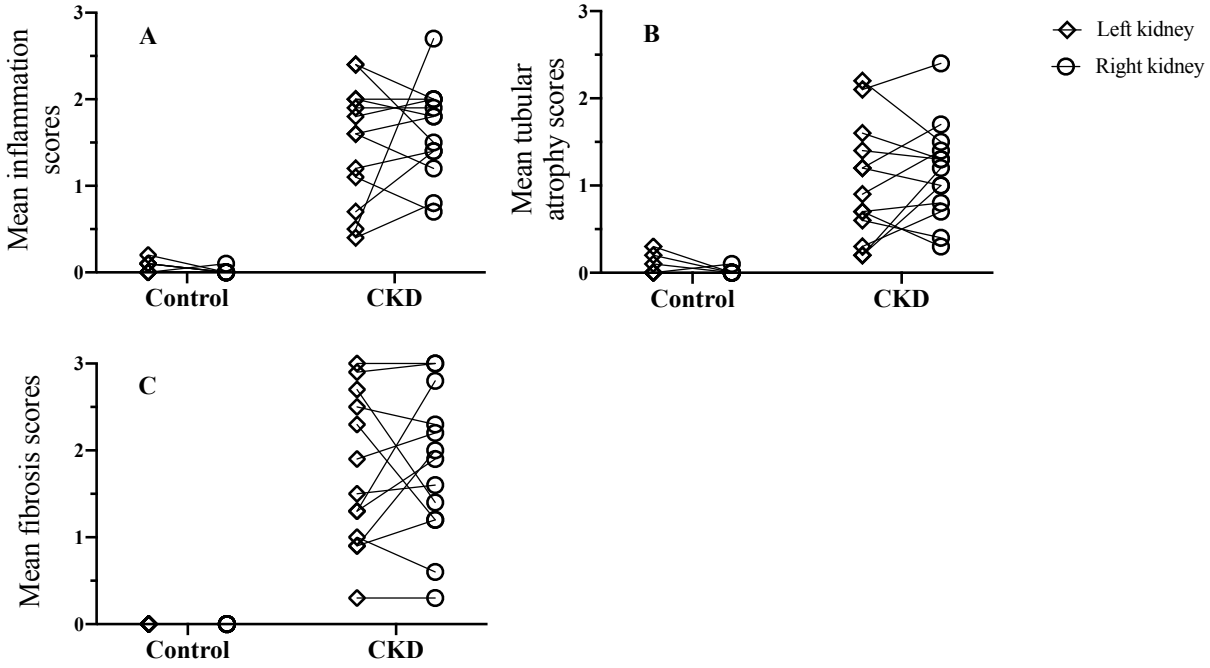

Figure 6.2. Dot plot of mean inflammation (A), tubular atrophy (B), and inflammation (C) scores of kidneys from cats with chronic kidney disease (n = 13) and from healthy adult control cats (n = 8). Values from the left (open diamond) and right kidney (open circle) of the same individual are connected by solid lines.

Correlation between gene transcript levels and histologic scores. There was a significant, positive association of TGF-β1 transcript levels and mean inflammation scores in the kidneys of CKD cats and all cats combined (Table 6.8), though the combined (both) group analysis is driven by the CKD slope.

Table 6.8. Tests of association of inflammation scores and gene transcript levels using the Pearson's correlation coefficient or a linear mixed model.

Gene	Group	r[a]	*P*-value[a]	Slope[b]	*P* -value[b]
HIF-1α	Control	0.40	0.127	1.91	0.390
	CKD	0.29	0.152	0.33	0.081
	Both	0.43	0.073	0.34	0.069
MMP-2	Control	0.07	0.785	0.79	0.699
	CKD	0.19	0.360	-0.02	0.901
	Both	0.82	0.276	-0.02	0.915
MMP-7	Control	-0.18	0.512	-1.30	0.706
	CKD	0.16	0.426	0.15	0.612
	Both	0.92	0.386	0.12	0.672
MMP-9	Control	0.08	0.769	0.05	0.986
	CKD	-0.09	0.650	-0.12	0.687
	Both	0.76	0.614	-0.12	0.687
TGF-β1	Control	-0.05	0.865	-0.12	0.944
	CKD	0.39	0.052	0.34	0.020
	Both	0.79	0.019	0.34	0.018
TIMP-1	Control	0.11	0.696	0.48	0.795
	CKD	0.16	0.437	0.04	0.824
	Both	0.83	0.328	0.04	0.819
VEGF-A	Control	0.50	0.050	2.10	0.079
	CKD	-0.35	0.079	-0.18	0.054
	Both	0.24	0.084	-0.17	0.084

[a] Pearson's correlation

[b] Linear mixed model with fixed effect = group, inflammation score, group*inflammation score; random effect = cat

There was also a significant, positive association of HIF-1α and TGF-β1 transcript levels and mean tubular atrophy scores in the kidneys of CKD cats and all cats (Table 6.9). Again, the combined (both) group analysis is driven by the CKD slope. There was a strong, positive association between MMP-7 transcript levels and tubular atrophy scores in the combined

analysis, but not within the control or CKD groups. There was a positive association of VEGF-A transcription and mean tubular atrophy score in the control group only, though this appeared to be driven by 2 data points.

Table 6.9. Tests of association of tubular atrophy scores and gene transcript levels using the Pearson's correlation coefficient or a linear mixed model.

Variable	Group	r[a]	*P*-value[a]	Slope[b]	*P*-value[b]
HIF-1α	Control	0.29	0.278	1.04	0.494
	CKD	0.36	0.073	0.42	0.033
	Both	0.46	0.026	0.43	0.027
MMP-2	Control	0.05	0.849	-0.18	0.901
	CKD	0.19	0.363	0.22	0.342
	Both	0.82	0.280	0.21	0.354
MMP-7	Control	-0.10	0.718	-0.67	0.771
	CKD	0.35	0.076	0.57	0.059
	Both	0.93	0.049	0.55	0.064
MMP-9	Control	-0.07	0.786	-1.06	0.619
	CKD	-0.23	0.262	-0.16	0.677
	Both	0.77	0.193	-0.18	0.623
TGF-β1	Control	0.09	0.730	0.22	0.849
	CKD	0.47	0.016	0.41	0.004
	Both	0.80	0.003	0.41	0.003
TIMP-1	Control	-0.01	0.976	0.15	0.911
	CKD	0.12	0.558	0.10	0.609
	Both	0.83	0.468	0.10	0.600
VEGF-A	Control	0.58	0.020	1.69	0.051
	CKD	-0.07	0.723	-0.04	0.692
	Both	0.17	0.872	-0.02	0.872

[a] Pearson's correlation, ignores within cat correlation

[b] Linear mixed model with fixed effect = group, tubular atrophy score, group*tubular atrophy score; random effect = cat

There was a significant, positive association of HIF-1α (Figure 6.3), MMP-2 (Figure 6.4) and MMP-7 (Figure 6.5) with mean fibrosis score in the kidneys of CKD cats (Table 6.10).

Table 6.10. Tests of association of fibrosis scores and gene transcript levels using the Pearson's correlation coefficient or a linear mixed model.

Variable	Group	r[a]	p-value[a]	Slope[b]	p-value[b]
HIF-1α	CKD	0.34	0.090	0.34	0.050
MMP-2	CKD	0.47	0.016	0.45	0.013
MMP-7	CKD	0.41	0.039	0.59	0.014
MMP-9	CKD	0.31	0.120	0.49	0.078
TGF-β1	CKD	0.32	0.117	0.22	0.098
TIMP-1	CKD	0.38	0.053	0.31	0.061
VEGF-A	CKD	-0.25	0.215	-0.09	0.215

[a] Pearson's correlation, ignores within cat correlation

[b] Linear mixed model with fixed effect = fibrosis score; random effect = cat

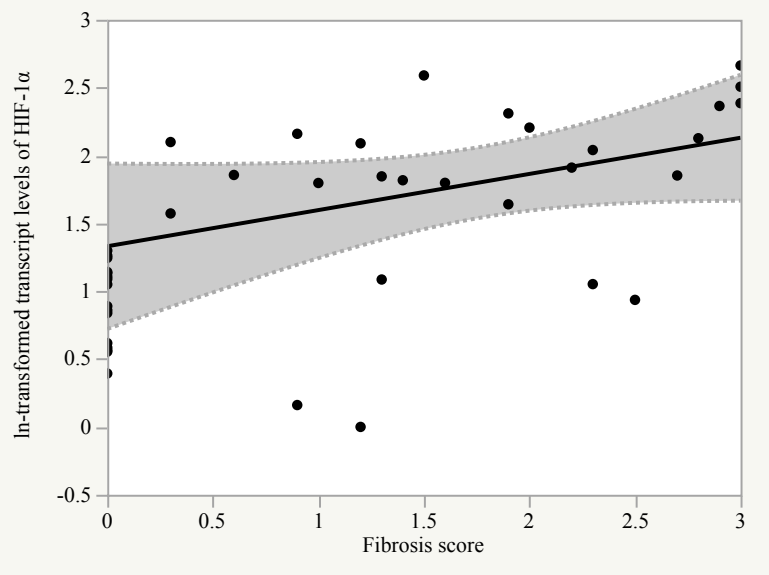

Figure 6.3. Scatter plot with line of best fit for mean fibrosis scores and natural log of gene transcript levels of HIF-1α. Dotted lines and shaded area represent the 95% confidence band of the regression line.

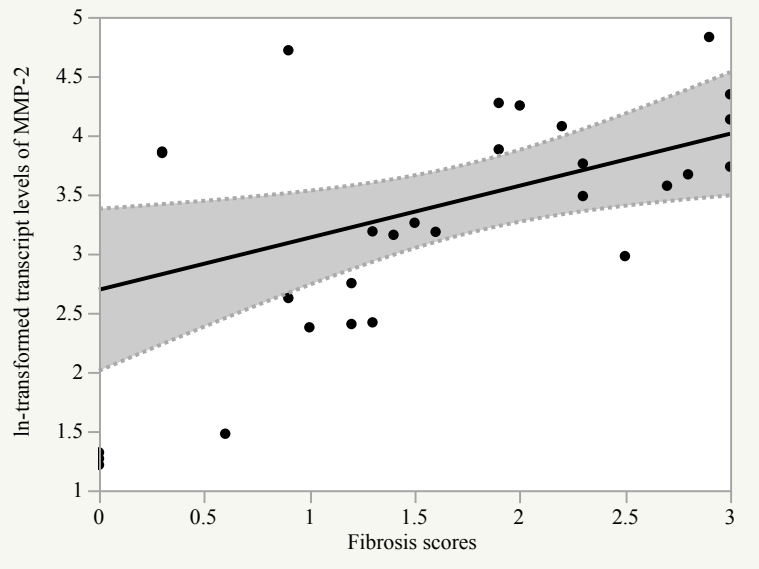

Figure 6.4. Scatter plot with line of best fit for mean fibrosis scores and natural log of gene transcript levels of MMP-2. Dotted lines and shaded area represent the 95% confidence band of the regression line.

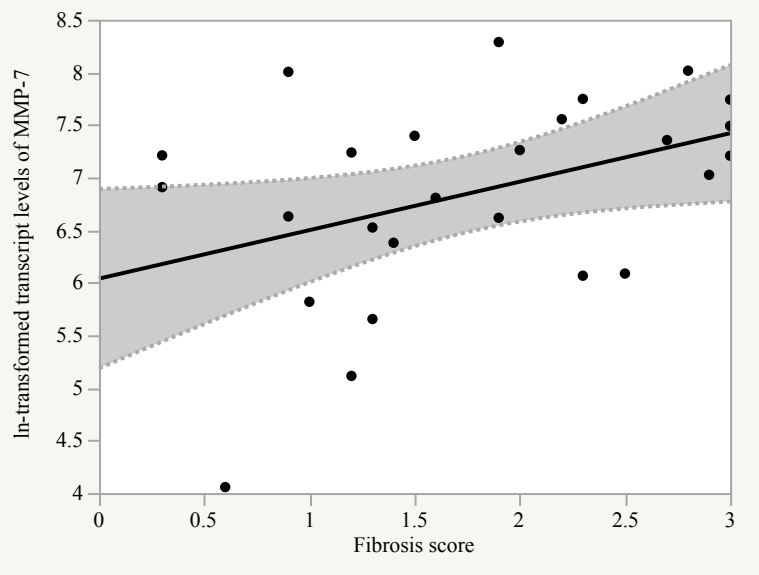

Figure 6.5. Scatter plot with line of best fit for mean fibrosis scores and natural log of gene transcript levels of MMP-7. Dotted lines and shaded area represent the 95% confidence band of the regression line.

Discussion

The present study shows upregulation of transcription of HIF-1α, MMP-2, MMP-7, MMP-9, TIMP-1 and TGF-β1, and downregulation of VEGF-A in the kidneys from cats with naturally occurring CKD when compared to those from healthy control cats. Work by our group has recently shown that these molecular pathways are similarly differentially transcribed in kidneys from cats subjected to transient renal ischemia as a model of CKD. Collectively, these data strengthen the evidence supporting the role of hypoxia in the intrinsic progression of CKD, and appoint specific candidates for biomarkers of tubulointerstitial inflammation and fibrosis in cats.

In tissues from cats with CKD, gene transcript levels of HIF-1α, MMP-2, MMP-7 and TGF-β1 were positively associated with worsening histologic lesion scores. Specifically, MMP-2 and MMP-7 were moderately correlated with worsening degrees of renal fibrosis. In human beings, MMP-7 is recognized as an important mediator of fibrosis in CKD, as well as a urinary biomarker of renal fibrosis.[103, 108, 272] The results of the present study suggest that MMP-7 may similarly represent a useful biomarker for this histologic pattern of change in cats; however, whether increased transcription of the MMP-7 gene is accompanied by increased urinary levels of this protein remains to be investigated.

Tubulointerstitial fibrosis is highly correlated with functional impairment.[185, 253] Thus, it was not surprising that transcript levels of these MMPs were also associated with sCr in the present study. Our results showed that transcription of MMP-2 and MMP-7 was positively and moderately correlated with sCr in cats from each group, whereas transcription of TIMP-1 was mildly correlated with sCr in CKD cats only. Serum concentrations of both MMP-2 and MMP-9, and their inhibitors TIMP-1 and TIMP-2 were increased in children with CKD when compared to age-matched controls, and these concentrations were elevated in proportion to disease stage.[107] Conversely, in adult human beings with CKD, serum activity of MMP-2 was increased, while activity of MMP-9 was decreased when compared to control subjects.[303] In that report, sCr directly correlated with MMP-2 activity but inversely correlated with that of MMP-9. Notably, seven of the cats in our study had atypical urothelial hyperplasia noted on histology. Studies in human beings document an association between increased urinary activities of MMP-2 and -9 and early-stage bladder carcinoma.[304] Balkan endemic nephropathy, a familial chronic tubulointerstitial disease characterized by tubulointerstitial nephritis in people with histologic features similar to those of feline CKD,[182, 269] is associated with the development of papillary

transitional cell carcinoma of the renal pelvis and/or urether.[305] If the urothelial changes observed in the cats in our studies represent preneoplastic lesions, it is possible that these lesions could have contributed to the observed upregulation of MMP-2 and -9. Nonetheless, an upregulation of these MMPs has been previously observed in cats with experimentally-induced ischemic CKD, which lacked atypical urothelial changes on histology.[a]

In a previous study, renal transcription of HIF-1α was not different between cats with experimentally-induced ischemic CKD and control cats; however, it positively correlated with worse scores of fibrosis in the affected kidneys.[a] In the present report, which evaluated cats with spontaneous CKD that were more severely histologically and functionally affected than those of the earlier study, a significance difference in transcription of this growth factor was indeed observed. Additionally, HIF-1α levels were positively correlated with both tubular atrophy and fibrosis scores. These data suggest chronic renal hypoxia as an ongoing feature of feline CKD and that HIF-1α transcription may be proportional to disease severity. Work performed in cultured renal cells and knockout mice models suggests that activation of HIF-1 signaling in renal epithelial cells is associated with the development of chronic renal disease by promoting fibrogenesis.[96, 258] Therefore, HIF-1α signaling may warrant evaluation as a therapeutic target in cats.

Transcription of TGF-β1 was noted to be upregulated in CKD and positively associated with worse inflammation and tubular atrophy scores in our study. Two prior reports document increased urinary TGF-β1 levels (expressed as urinary TGF-β1-to-creatinine ratio) in cats with naturally occurring CKD, as compared to healthy control cats.[204, 205] In the most recent, a positive correlation between this ratio and sCr was identified.[205] Similar correlation was not observed in the present study and, interestingly, although TGF-β1 is a prototypical fibrogenic

cytokine, there was no significant correlation between its transcript levels and the histologic scores for fibrosis. As regulation of expression of TGF-β1 occurs at both the transcription and translation steps,[306] it is possible that assessment of transcript levels may not directly reflect levels of TGF-β1, which may have influenced our results.

In addition to documenting increased urinary TGF-β1 levels in cats with CKD, Habenicht and colleagues noted that cats with CKD had significantly lower urinary VEGF levels than did normal cats.[205] These findings are in accordance with those of the present study. It has been hypothesized that decreased VEGF may promote loss of peritubular capillary density and contribute to progressive renal disease.[120] Nevertheless, the role of this growth factor in the pathophysiology of renal disease is complex. Conflicting data from different models and distinct forms of renal disease describe it as deleterious in some disease settings and protective in others.[307]

There are limitations to this study. First, cats with CKD were significantly older and more likely to be sexually-altered (via orchiectomy or ovariohysterectomy) than control cats. While age was not significantly associated with the transcript levels of any gene in our study, the impact of neutering was not explored. Further, as post-transcriptional regulation of gene expression was not examined, any impact of age or sex in that portion of regulation of gene expression would have been missed. Additionally, cats with CKD were obtained from a population of client-owned cats whereas control cats were obtained from feral or purpose-bred cat populations. Therefore, differences in diet and environment were likely. Of relevance, as this study sought to characterize renal transcription, tissue samples were required. Because these were procured after death or euthanasia, a bias towards animals who may have been experiencing relatively rapid disease progression is likely in the CKD group. To this point, acute

exacerbation of renal disease was not able to be distinguished from stable CKD in all cases. It is possible that an overrepresentation of acute-on-chronic disease biased our results towards patterns observed in acute kidney injury. Finally, as blood and urine sampling was not performed in client-owned cats, laboratory data from cats with CKD was collected at various time points before euthanasia. Thus, these data may not accurately reflect disease state at the time of collection, either by representing an earlier timepoint, or by being acquired during a period of decompensation, when the patient was likely to be experiencing pre-renal azotemia. The potential impact of these factors must be considered when interpreting the reported correlations between gene transcript levels and sCr.

In conclusion, kidneys from cats with CKD show upregulation of transcription of pro-fibrotic cytokines and growth factors known to be induced by ischemic injury. Future evaluation of HIF-1α, MMP-2, MMP-7 and TGF-β1 is warranted to explore their potential as biomarkers of renal fibrosis and as targets for therapeutic intervention in feline CKD.

Footnotes:

a. Lourenço BN, Coleman AE, Schmiedt CW, et al. Characterization of hypoxia-induced, profibrotic pathways in an ischemic model of feline chronic kidney disease (abstract), in *J Vet Intern Med*. 2018;32:2278.

b. Lourenço BN, Tarigo JL, Stanton JB, et al. Inflammatory and pro-fibrotic pathway upregulation in an ischemic model of feline chronic kidney disease (abstract), in *J Vet Intern Med*. 2019;33:1052.

c. SNAP FIV/FeLV Combo Test, IDEXX Laboratories, Westbrook, ME.

d. IDEXX Laboratories, Inc., Westbrook, ME.

e. RNAlater stabilization solution, QIAGEN, Valencia, CA.

f. RNeasy Plus Mini Kit, QIAGEN, Valencia, CA.

g. NanoDrop Spectrophotometer, Thermo Fisher Scientific, Waltham, MA.

h. ezDNase, Invitrogen, Carlsbad, CA.

i. SuperScript IV VILO Master Mix, Invitrogen, Carlsbad, CA.

j. SsoAdvanced SYBR Green Supermix, Bio-Rad Laboratories, Hercules, CA.

k. CFX96, Bio-Rad Laboratories, Hercules, CA.

l. PGEM-T easy vector, Promega, Madison, WI.

m. Molecular Cloning Laboratories, San Francisco, CA.

n. SAS, version 9.3, SAS Institute Inc, Cary, NC.

o. GraphPad Prism for Mac, version 7, GraphPad Software Inc, La Jolla, CA.

CHAPTER 7

CHARACTERIZATION OF THE INTRARENAL AND CIRCULATING RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM IN EXPERIMENTAL AND NATURALLY-OCCURRING FELINE CHRONIC KIDNEY DISEASE[5]

[5] Bianca N. Lourenço, Amanda E. Coleman, Jaime L. Tarigo, DVM, Cathy A. Brown, Daniel R. Rissi, Chad W. Schmiedt, and Scott A. Brown. To be submitted to the *Journal of Veterinary Internal Medicine*.

Abstract

Background: The role of the renin-angiotensin-aldosterone system (RAAS) in the pathogenesis of feline chronic kidney disease (CKD) is undefined.

Objective: To characterize the intrarenal renin-angiotensin system (RAS) in feline CKD, and to describe the relationship between intrarenal, circulating and urinary RAAS markers.

Animals: Thirteen cats with naturally-occurring CKD (natCKD), 11 cats with experimentally-induced CKD (representing two models), and 16 healthy control cats (8 juvenile and 8 adult).

Methods: Expression of intrarenal RAS components was characterized in renal tissues from all cats using reverse-transcription polymerase chain reaction and liquid chromatography-mass spectrometry techniques, and compared among groups. Plasma renin activity (PRA) and urinary aldosterone-to-creatinine ratio (UACR) were measured in the two CKD model and control cats, and evaluated for association with expression of intrarenal RAS components. Linear mixed models and the Pearson's correlation coefficient were used for statistical analyses at a significance level of 0.05.

Results: Compared to control kidneys, tissues from natCKD and one model of CKD showed significantly higher renal transcript levels of angiotensinogen ($P < 0.001$) and lower transcript levels of angiotensin converting enzyme ($P < 0.001$). Renal angiotensin I, II and III concentrations were significantly different among groups. Angiotensin I concentrations were increased in natCKD compared with juvenile and adult controls ($P < 0.001$). Measurements of PRA and UACR were correlated with intrarenal angiotensin peptide concentrations in one model of CKD, but not the other or either of the control groups.

Conclusion and clinical significance: The intrarenal RAS appears to be overstimulated in feline CKD. Markers of the circulating RAAS are poor indicators of tissue RAS component levels in cats.

Abbreviations

ACE, Angiotensin-converting enzyme

AGT, Angiotensinogen

Ang, Angiotensin

AT_1R, Angiotensin II subtype 1 receptor

CKD, Chronic kidney disease

GFR, Glomerular filtration rate

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

LC-MS/MS, Liquid chromatography-mass spectrometry

LMM, Linear mixed model

natCKD, Naturally-occurring CKD

PRA, Plasma renin activity

RI, Renal ischemia

RI-DCN, Renal ischemia followed by delayed contralateral nephrectomy

RPS7, Ribosomal protein S7

UACR, Urinary aldosterone-to-creatinine ratio

Introduction

Ample evidence incriminates the renin-angiotensin-aldosterone system (RAAS) as a critical factor in the pathophysiology of the progression of chronic kidney disease (CKD),[308, 309] making RAAS inhibition an integral part of the treatment of renal disease in human beings.[161] Studies characterizing this system in feline CKD have yielded conflicting results. Those examining cats with naturally-occurring CKD and systemic hypertension demonstrated lower mean plasma renin activity (PRA) and higher mean plasma aldosterone concentration, in affected cats when compared with controls,[209, 211, 212] although both PRA and aldosterone levels were (inappropriately) within or above the reference range in a subset of these patients. When the RAAS was studied in experimentally-induced CKD, both PRA and circulating aldosterone concentrations were increased in some, but not all, of the described models.[207, 208]

Recently, focus on the role of the RAAS in health and disease states has been extended to that of the local, tissue renin-angiotensin systems (RAS).[45] Work in rodent models has established that all RAS components can be produced in the kidney, allowing formation of intrarenal angiotensin II (AngII) by local mechanisms that appear to be subject to feedback mechanisms that are independent of the traditional, circulating RAAS.[41, 42, 46, 49] Limited work has characterized intrarenal RAS in companion animal species. One study reported that tubular and interstitial AngII immunoreactivity correlated with glomerulosclerosis and cell infiltration, but not tubulointerstitial fibrosis, in cats.[152] Most other reports to date have evaluated components of the circulating, systemic RAAS, leaving the importance of the tissular system largely undescribed.

The main objective of this study was to characterize the tissue expression of intrarenal RAS components in naturally-occurring and experimentally-induced feline CKD, and in healthy

individuals. We hypothesized that compared to tissues from normal control cats, those from cats affected by spontaneous or experimentally-induced CKD would demonstrate increased transcription of genes encoding for proteins of the RAS and increased concentrations of angiotensin peptides. A secondary objective was to evaluate the correlation between markers of the circulating RAS, here PRA and urinary aldosterone-to-creatinine ration (UACR), and expression of intrarenal RAS components. We hypothesized that markers of the circulating and intrarenal systems would be poorly correlated, suggesting independent regulation of the local and circulating systems.

Materials and methods

Study design. This study was performed on banked plasma, urine, and/or renal tissue samples collected from five populations: 1) client-owned cats with naturally-occurring CKD (group natCKD; n = 13), 2) cats having previously undergone unilateral, *in vivo* renal ischemia as a model of CKD (group RI; n = 6), 3) cats having previously undergone unilateral, *in vivo* renal ischemia followed by delayed, contralateral nephrectomy as a model of CKD (group RI-DCN; n = 5), 4) healthy, adult control cats without evidence of CKD (group adult control; n = 8), and 5) healthy, juvenile, female intact cats without evidence of CKD (group juvenile control; n = 8). An overview of the origin of the cats and respective samples evaluated in the study is presented in table 7.1.

Table 7.1. Overview of provenance and type of banked samples available from cats with naturally-occurring chronic kidney disease, experimental chronic kidney disease, and healthy control cats enrolled in the present study.

Group	Origin	Samples available
natCKD	Primary care and referral hospitals	Renal tissues
RI	Purpose-bred cats	Plasma, urine and renal tissues
RI-DCN	Purpose-bred cats	Plasma, urine and renal tissues
Adult control	Animal control facility Purpose-bred cats	Renal tissues Plasma, urine and renal tissues
Juvenile control	Purpose-bred cats	Plasma, urine and renal tissues

Abbreviations: natCKD, naturally-occurring chronic kidney disease; RI, renal ischemia, RI-DCN, renal ischemia-delayed contralateral nephrectomy.

Renal tissue samples from all cats were used to compare transcript levels of the RAS genes angiotensinogen (AGT), renin, angiotensin-converting enzyme (ACE) and angiotensin II subtype 1 receptor (AT_1R), and the concentration of renal angiotensin I, II, III and IV peptides between groups. Available plasma and urine samples from the two CKD models (RI and RI-DCN) and the two control groups (adult and juvenile controls) were used to measure markers of the circulating RAAS (i.e., PRA and UACR) and correlate those with intrarenal RAS measurements. For the two CKD model groups, circulating markers of RAAS activation were measured in samples collected before and after renal injury, allowing for comparison of measurements obtained at different time points.

Animals. Samples from natCKD, RI, adult control and juvenile control groups were obtained from the cats described in chapters 4, 5 and 6 of the present dissertation. Briefly, renal tissues from the natCKD group were obtained from client-owned cats presenting to primary care and referral veterinary centers for humane euthanasia. These samples were obtained and banked as part of a study designed to investigate pathways of renal fibrosis in feline CKD. Informed

consent was obtained prior to post-mortem collection of both kidneys. Blood and urine samples from these cats were not available.

Samples for the juvenile control group were obtained from purpose-bred, clinically healthy, mixed breed, intact female cats that were previously enrolled in unrelated terminal studies with no impact on renal structure and function. For the adult control group, samples were similarly recruited from adult purpose-bred cats enrolled in prior unrelated, terminal studies, as well as from adult cats that were euthanized as part of population control measures at a local animal control facility. Cats obtained from the feral population had no evidence of exposure to retroviral infections based on the results of feline immunodeficiency virus antibody and feline leukemia virus antigen testing.[c]

Samples from the two experimentally-induced CKD groups were banked from the original studies intended for development the respective disease models. The RI group included six purpose-bred female spayed cats that completed a prior study assessing the chronic effects of unilateral, transient renal ischemia on renal function and histology.[200] Briefly, these cats underwent 90-minutes of unilateral renal ischemia, applied to the right kidney, and were followed for a period of six months. Data reflecting renal function, including glomerular filtration rate (GFR), serum creatinine (sCr), serum urea nitrogen (SUN), urine specific gravity (USG) and urine protein-to-creatinine ratio (UPC), and indirect systolic blood pressure measurements, were collected through that study and have been described elsewhere. The RI-DCN group was comprised of five purpose-bred male cats that completed a separate CKD model study evaluating the chronic effects of transient unilateral renal ischemia, followed by delayed, contralateral nephrectomy. These cats underwent anesthesia for 90-minute occlusion of the right renal artery and vein using the previously described technique,[200] and were allowed to recover

from the ischemic injury. Three months after unilateral renal ischemia (day 90), the cats were anesthetized for nephrectomy of the contralateral, unmanipulated (left) kidney. The cats were followed for a period of six months after contralateral nephrectomy (nine months post-ischemic injury), and were then euthanized for collection of the previously ischemic kidney (day 270). One cat met that study's predetermined endpoint for humane euthanasia for compassionate care and was euthanized at day 93 due to severe azotemia (i.e., sCr > 8mg.dL). The remaining four cats were followed for the full 270 day-study period. Similar to the previous CKD model study, data reflecting renal function, including GFR, sCr, SUN, USG and UPC, were collected for these cats. In addition, direct systemic arterial blood pressure measurements were obtained at various timepoints using telemetric implants placed in the femoral artery. Timing of plasma and urine sampling, surgical interventions, and banking of biological samples in these two studies is summarized in figure 7.1.

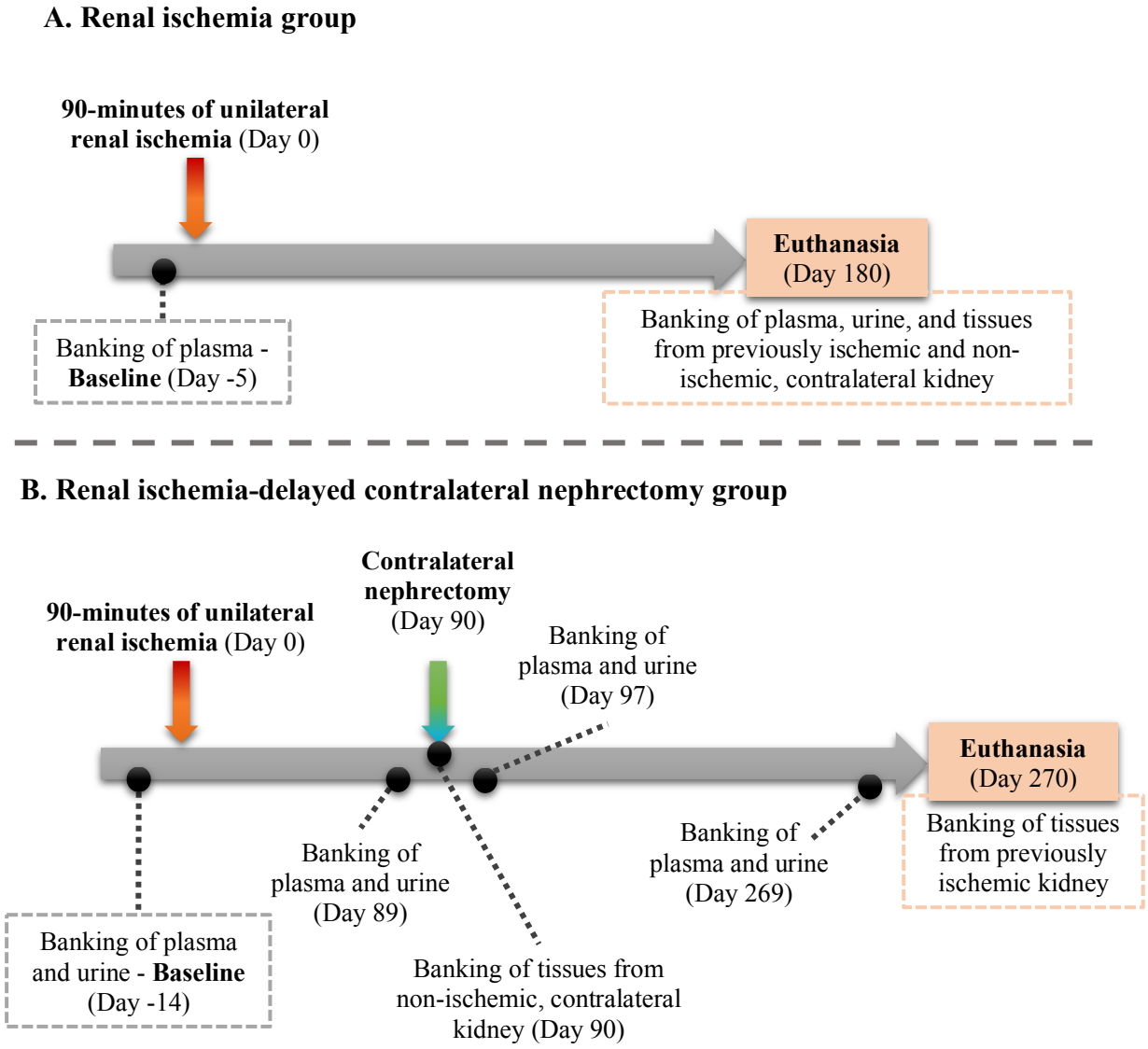

Figure 7.1. Overview of sample collection for cats who underwent transient, unilateral renal ischemia (RI group, n = 6; panel A) or unilateral, transient renal ischemia followed by delayed, contralateral nephrectomy (group RI-DCN, n = 5; panel B) as a model of chronic kidney disease.

Plasma, serum and urine sample collection and processing. Blood for plasma samples was collected into tubes containing EDTA as an anticoagulant, and immediately placed on ice.

Plasma was separated under refrigeration at 4°C. For serum samples, blood was collected into glass tubes and separated after clot formation. Urine was collected into glass tubes and immediately placed on ice. All biological samples were aliquoted and stored at -80°C until analysis. Samples analyzed by external laboratories were batched and shipped on dry ice.

Markers of circulating RAAS. Plasma renin activity was measured on EDTA-preserved plasma from CKD model and control cats. Renin activity measurement was performed by a commercial laboratory,[a] using a liquid-chromatography-mass spectrometry (LC-MS)-based method as described previously.[310] Briefly, feline EDTA-plasma was thawed and diluted in an AngI generation buffer containing ethylenediaminetetraacetic acid[b] (5μM), Z-Pro-Prolinal[c] (20 μM), 4-(2-Aminoethyl)benzene-sulfonyl fluoride hydrochloride[d] (1mM) and aminopeptidase inhibitor[e] (10 μM) in phosphate-buffered saline.[f] Mixtures were split into two aliquots, one of which was placed on ice and used as baseline control while the second was incubated at 37°C for 1 hour. Following incubation, the mixtures were stabilized and further spiked with stable isotope labelled internal standard for AngI at a concentration of 200 pg/ml. The stabilized samples then underwent C-18-based solid-phase-extraction and were subjected to LC-MS/MS analysis using a reversed-phase analytical column operating in line with a mass spectrometer.[g] Internal standard was used to correct for peptide recovery of the sample preparation procedure in each individual sample. Plasma renin activity was calculated by subtracting AngI formation in incubated aliquot from the aliquot on ice (baseline control) and expressed as (ng/ml)/h.

Urinary aldosterone-to-creatinine ratio measurements were performed by an external laboratory.[h] Urinary aldosterone was measured using a commercially available radioimmunoassay kit.[i] Urinary creatinine was measured using the modified Jaffe method. The

UACR was calculated as urinary aldosterone concentration (pmol/L) divided by the urinary creatinine concentration (mmol/L).

Renal tissue sample collection. For all cats, renal tissues were collected within one hour of euthanasia. Kidneys were removed through a midline laparotomy and sectioned longitudinally. One-half of each kidney was minced and placed in RNA stabilization solution,[j] and the remaining portion was placed in neutral-buffered 10% formalin. Following overnight incubation at 4°C, tissues were removed from RNA stabilization solution, homogenized with a mortar and pestle, divided into 30 mg aliquots, and stored at -80°C until further analysis.

Renal RAS gene transcription. For each sample, total RNA was extracted from 30 mg of tissue homogenate using a commercially available RNA extraction kit.[k] Integrity of the isolated RNA was confirmed by quantification using a spectrophotometer,[l] followed by visualization of 18S and 28S ribosomal bands on 1.2% agarose gels. A total of 1 µg of RNA extracted from each sample was treated with deoxyribonuclease,[m] and reverse-transcribed using a cDNA reaction master mix.[n] Quantification was performed in an automated cycler[o] using 20 µL reactions containing 10 µL of SYBR Green Supermix,[p] 5 pmol of each primer (Table 7.2), and 9 µL of complementary DNA sample at a 1:40 dilution. Thermal cycling conditions consisted of an activation step at 95°C for 30 seconds, followed by 36 amplification cycles (95°C for 15 seconds for denaturation and 60°C for 30 seconds for annealing and extension), and a melt curve step (60°C to 95°C, increasing at increments of 0.5°C every 5 seconds). All reactions were performed in triplicate and average values were used for further analyses. A no reverse transcriptase control was included for each sample and three no template controls were used in each plate. In accordance with the sample maximization method,[259] for each gene, all samples were analyzed in two 96-well plates. A standard sample was analyzed in triplicate with glyceraldehyde 3-

phosphate dehydrogenase (GAPDH) primers in each plate and used as an inter-run control for the entire gene study.

Table 7.2. Primer sequences for quantitative polymerase chain reaction.

Gene	Ensemble ID/ NCBI Access. No.	Primer sequence (5'→ 3')	Fragment size (bp)	Source
GAPDH	ENSFCAG 00000006874	for: GCTGCCCAGAACATCATCC rev: GTCAGATCCACGACGGACAC	134	Riedel et al.[260]
RPS7	NM_001009832	for: GTCCCAGAAGCCGCACTT T rev: CACAATCTCGCTCGGGAA AA	74	Kessler et al.[261]
AGT	XM_011287235.1	for: TCT TGA CGT GGC TGA AGA ATC rev: AGC AGG TCC TGA AGG TCA TA	94	IDT PrimerQuest[311]
Renin	ENSFCAG 00000002902	for: CGA CCC CCA GTA TTA CCA AG rev: CCT CCT CAC AGA CCA AGG TG	116	Riedel et al.[260]
ACE	XM_003997083.1	for: GCT GAA ACC CCT GTA CCA GA rev: GAG GTG TTC CAG ATC CTC CA	133	Riedel et al.[260]
AT_1R	ENSFCAT00000000716	for: AAG ATC GCT TCA GCC AGT GT rev: TTG GCC ATA AGC ATT GTA CG	134	Riedel et al.[260]

Abbreviations: AGT, angiotensinogen; ACE, angiotensin-converting enzyme; AT_1R, angiotensin II subtype 1 receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RPS7, ribosomal protein S7

Transcript levels of the target genes of AGT, renin, ACE and AT_1R were normalized to those of two reference genes, GAPDH and ribosomal protein S7 (RPS7), using the GENorm method.[264] Reference genes were selected based on the efficiency of their primers, and their stability value (M value < 0.5) and coefficient of variance (< 0.25) when tested in a complete set of the experiment's samples. After normalization, transcript levels were scaled to those of the lowest sample for each target gene.

Gene specific primers were selected from previously reported studies, when available, or designed using the IDT PrimerQuest tool[311] (Table 7.2). Primer efficiencies were calculated

using the Pfaffl method based on standard curves performed with serial dilutions of cDNA (factor of two or four; three replicates) starting at a 1:20 dilution. Quantitative PCR products were confirmed on 1.2% agarose gels. Amplicons were cloned into a vector system[q] and sequenced bi-directionally by chain termination.[r] Resulting sequences were confirmed via NCBI-BLAST against the feline genome.[265]

Renal angiotensin peptides. The intrarenal angiotensin peptides AngI, AngII, AngIII and AngIV were measured on RNA*later* preserved renal tissue homogenates by a commercial laboratory[a] using the LC-MS-based technique RAS-Fingerprint® as described previously.[312] Briefly, frozen renal tissue samples (80 - 100 mg) were homogenized using pestle and mortar under liquid nitrogen. The frozen tissue powder was dissolved at 100 mg/ml in 6 mol/l aqueous guanidinium chloride[s] supplemented with 1% (v/v) trifluoroacetic acid[t] by cooled sonication using a 2mm microtip.[u] Stable isotope-labelled internal standards for individual angiotensin metabolites were added to tissue homogenates at 200 pg/ml and stored at -80 °C until analysis. Liquid chromatography-mass spectrometry/mass spectrometry analysis for tissue angiotensin quantification was performed in the same way as for PRA analysis.

Statistical analyses. Statistical analyses were performed using commercially available software packages.[v,w] A significance threshold of 0.05 was used.

The distributions of clinical variables and PRA and UACR data by study group were examined for normality by visual assessment of histograms and normal quantile plot, and the Shapiro-Wilk test. Normally distributed data are presented as mean ± SD and compared between groups using a one-way ANOVA test, with pairwise comparisons adjusted for multiple comparisons using the Dunnett's test. Non-normally distributed data are presented as median (range) and compared between groups using the Kruskal-Wallis test, with subsequent pairwise

comparisons performed using the Steel-Dwass test, therefore adjusting for multiple comparisons. For purposes of statistical analyses, USG values reported as >1.060 and SUN values reported as >100 mg/dL were assigned a value of 1.061 and 101, respectively.

Histograms and Q-Q plots of renal scaled normalized gene transcript levels, renal angiotensin peptide concentrations, PRA, and UACR linear mixed model (LMM) residuals were examined to evaluate the assumption of normality. Model residuals were right-skewed and unequal between groups. Therefore, renal scaled normalized gene transcript levels, renal angiotensin peptide concentration, PRA, and UACR levels were natural log transformed prior to analysis, which improved the normality of the residuals and homogeneity of variances. All LMMs included a random factor of cat to account for within cat correlation. Satterthwaite degrees of freedom method and restricted maximum likelihood estimation were used in all LMMs. Linear mixed models were used to compare kidney natural log-transformed scaled normalized gene transcript levels and angiotensin peptides between groups. The full model for each gene transcript or angiotensin peptide variable had a single fixed factor for group. Multiple comparisons were adjusted for using Tukey's test.

Pearson's correlations were used to test for association of PRA or UACR with natural log transformed kidney scaled normalized gene transcript levels and angiotensin peptide concentrations of renal samples for the RI, RI-DCN, adult control and juvenile control groups separately, and for all cats in these groups in conjunction. Data were used to measure association of intrarenal and circulating RAS markers if collected within the same 24-hour window (e.g., for the RI-DCN group, PRA and UACR measured at day 89 were tested against intrarenal transcript levels and angiotensin peptide concentrations from kidney samples from day 90). For the RI group both kidneys were sampled concurrently with urine and plasma at the time of euthanasia.

When all cats were included in the analysis, PRA and UACR measurements of the RI group cats were assessed for association with the intrarenal RAS expression in the ischemic kidney, while the data from the non-ischemic kidney was excluded for analysis, which improved the measure of association.

Linear mixed models were also used to compare PRA and UACR before and after experimentally-induced renal injury for the RI and the RI-DCN groups separately. The full model for each marker of the circulating RAS included a fixed factor for timepoint. Multiple comparisons were adjusted for using Dunnett's test. Timepoint "Day 93" was not included in the analysis, as there was only one cat evaluated.

Results

Animals and samples. A total of 71 kidneys from 40 cats were evaluated in the present study (Figure 7.2). Transcript levels of genes encoding RAS proteins were measured in all available renal samples, and angiotensin peptides were measured in all but the left kidneys of adult control cats.

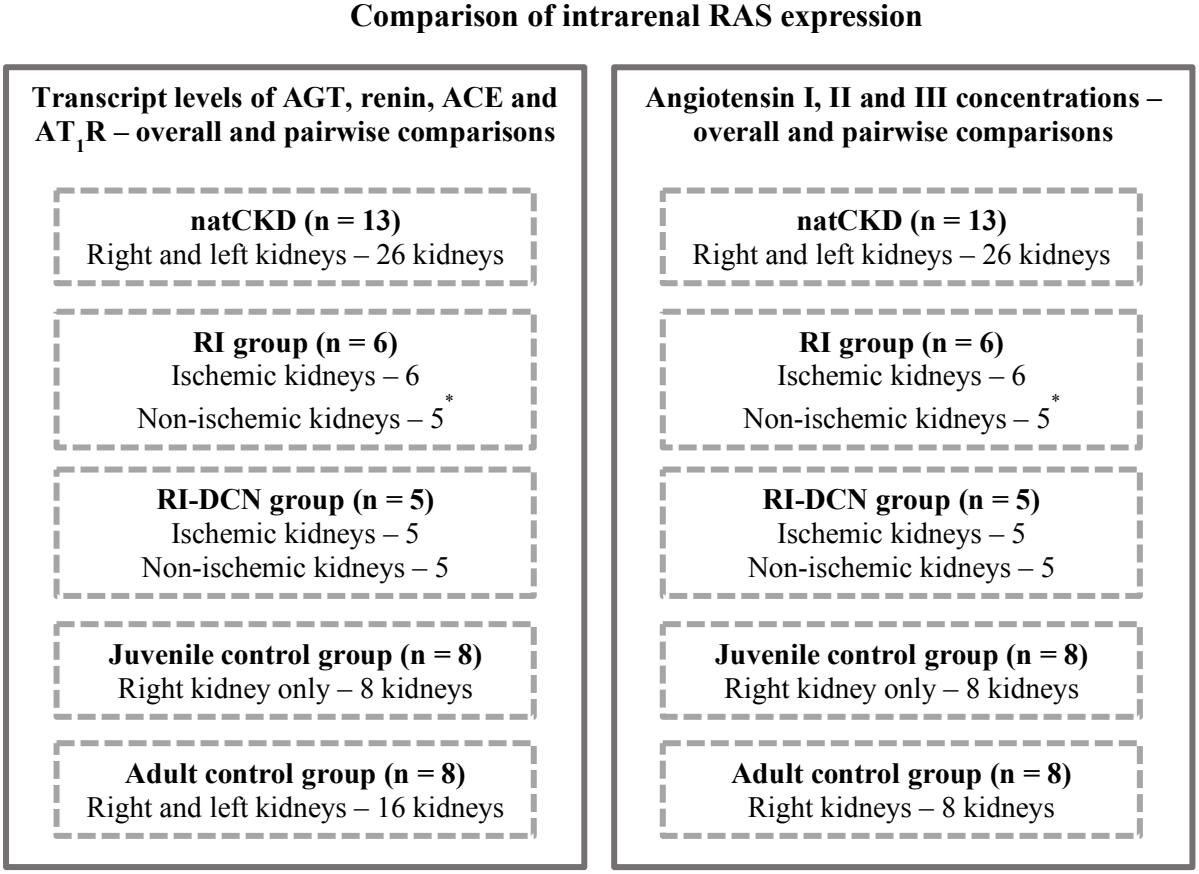

Figure 7.2. Overview of comparisons of intrarenal renin-angiotensin system expression among groups in the present study.

Abbreviations: natCKD, naturally-occurring chronic kidney disease; RAS, renin-angiotensin system; RI, renal ischemia; RI-DCN, renal ischemia-delayed contralateral nephrectomy.

*For one non-ischemic kidney, quality of banked sample was insufficient for analyses.

Plasma and urine samples were available for all cats in the RI-DCN group, and for a subset of cats in the RI, adult control and juvenile control groups, allowing for measurement of PRA and UACR. Table 7.3 summarizes the number of cats for which PRA and UACR were measured at each time point, and whether those were used for investigation of association with intrarenal RAS expression.

Table 7.3. Number of cats in each group for which plasma renin activity and urinary aldosterone-to-creatinine ratio measurements were available at each timepoint, and intrarenal renin-angiotensin system expression information used to investigate the association between the circulating and intrarenal systems.

Group	N	Timepoint	Circulating RAAS	Intrarenal RAS
RI model	6	Baseline	PRA (n = 6)	
		Day 180	PRA (n = 6) UACR (n = 3)	RAS gene transcript levels of BK Ang peptide concentrations of BK
RI-DCN model	5	Baseline	PRA (n = 5) UACR (n = 5)	
		Day 89	PRA (n = 5) UACR (n = 5)	RAS gene transcript levels of NIK Ang peptide concentrations of NIK
		Day 93*	PRA (n = 1) UACR (n = 1)	
		Day 97	PRA (n = 4)	
		Day 269	PRA (n = 4) UACR (n = 4)	RAS gene transcript levels of IK Ang peptide concentration of IK
Juvenile control	8	Euthanasia	PRA (n = 8) UACR (n = 7)	RAS gene transcript levels of RK Ang peptide concentrations of RK
Adult control	8	Euthanasia	PRA (n = 4) UACR (n = 3)	RAS gene transcript levels of BK Ang peptide concentrations of RK

Abbreviations: BK, both kidneys; IK, previously ischemic kidney; NIK, non-ischemic, contralateral kidney; PRA, plasma renin activity; RAS, renin-angiotensin system; RAAS, renin-angiotensin-aldosterone system; RI, renal ischemia; RI-DCN, renal ischemia-delayed contralateral nephrectomy; RK, right kidney; UACR, urinary aldosterone-to-creatinine ratio.

* One cat in the RI-DCN group met the study endpoint for humane euthanasia (i.e., serum creatinine concentration > 8.0 mg/dL) and was euthanized at day 93.

Demographic and clinical information for the cats included in the present study is presented in table 7.4. There were overall differences in median age ($P < 0.001$), sex distribution ($P < 0.001$) and mean body weight between all groups ($P = 0.006$).

Table 7.4. Demographic and clinical information from cats with naturally-occurring and experimentally-induced chronic kidney disease, and healthy control cats included in the present study. Data are presented as mean ± SD or median (range) where appropriate.

	natCKD	**RI**[†]	**RI-DCN**	**Juvenile control**	**Adult control**
Number included	13	6	5	8	8
Age (years)	17.3 (3.1-19.4)	1.4 (1.3-1.6)	2.4 (1.2-2.4)	0.7 (0.6-0.8)	1.7 (1-7)
Sex					
Female spayed	7	6	0	0	0
Female intact	0	0	0	8	3
Male neutered	6	0	5	0	4
Male intact	0	0	0	0	1
Breed					
Domestic short haired	9	6	5	8	8
Domestic medium haired	3	0	0	0	0
Abyssinian	1	0	0	0	0
Body weight (kg)	3.85 ± 1.37	4.64 ± 0.62	5.72 ± 0.99	3.48 ± 0.67	4.22 ± 0.95
n	11	6	5	8	8
sCr (mg/dL)	4.8 ± 3.4 3.7 (0.8-10.7)	1.4 ± 0.1 1.4 (1.3-1.6)	3.7 ± 3.4 1.6 (1.6-9.3)	1.0 ± 0.1 1.0 (0.8-1.2)	0.9 ± 0.5 0.9 (0.2-1.6)
SUN[*] (mg/dL)	73.9 ± 33.7 71 (28-14)	24.0 ± 1.8 23.5 (22-22)	42.8 ± 29.8 30.5 (23-87)	27.0 ± 3.8 27 (23-34)	24.9 ± 5.1 26.5 (15-30)
n	13	6	4	8	8
SDMA (µg/dL)	12.0 ± 5.4 9.5 (9-20)	8.2 ± 1.7 8 (6-10)	N/A	12.4 ± 2.7 12 (9-16)	10.3 ± 2.4 10.5 (6-14)
n	4	6	0	8	8
USG[**]	1.025 ± 0.019 1.018 (1.011-1.061)	1.054 ± 0.007 1.056 (1.041-1.059)	1.032 ± 0.014 1.032 (1.012-1.047)	1.052 ± 0.008 1.053 (1.037-1.061)	1.051 ± 0.007 1.049 (1.040-1.061)
n	9	6	5	8	8
UPC	0.69 (0.39-23.41)	0.10 (0.05-0.11)	0.14 (0.08-0.31)	0.14 (0.1-0.2)	0.19 (0.07-0.41)
n	4	6	4	8	8

Abbreviations: N/A, not available; natCKD, naturally-occurring chronic kidney disease; RI, renal ischemia model, RI-DCN, renal ischemia-delayed contralateral nephrectomy model; sCr, serum creatinine concentration; SDMA, serum symmetric dimethylarginine concentration; SUN, serum urea nitrogen; UPC, urinary protein-to-creatinine ratio; USG, urine specific gravity.
For ease of comparison, both mean and median are presented for variables with discordant assessment for approximation to normal distribution between groups.
[†]For the renal ischemia group, values for biochemical variables represent the average of two measurements obtained within 4 days of euthanasia.
[*]Serum urea nitrogen concentrations reported as > 100 mg/dL were assigned a value of 101 mg/dL.
[**]Urine specific gravity values reported as > 1.060 were assigned a value of 1.061.

Information regarding dietary sodium intake was known for the majority of cats with natCKD, half of the adult control cats, and all cats in the RI and RI-DCN groups. Cats in the RI and juvenile control groups, and four cats in the adult control group were being fed maintenance cat chow with a sodium content of 153-165 mg/100 kcal. Cats in the RI-DCN group were initially being fed maintenance cat chow (153 mg sodium/100 kcal) and were transitioned to a maintenance diet with a sodium content of 66 mg/100 kcal two weeks after renal ischemia. In the natCKD group, seven cats were being fed a prescription renal diet (Hill's k/d in six cases [56 mg sodium / 100 kcal] and Royal Canin Feline Renal Support D [90 mg sodium / 100 kcal] in the remaining case). One cat was being fed a maintenance diet (Pro Plan Focus urinary tract formula [60 mg sodium / 100 kcal in the dry formula and 94 mg sodium / 100 kcal in the canned formula]). Dietary information was not available for five natCKD cats.

One cat in the natCKD group was receiving atenolol prior to euthanasia. No other cats included the present study were being administered medications with known impact on the RAAS (e.g., calcium channel blockers or furosemide).

Intrarenal RAS gene transcription. There were statistically significant differences in gene transcript levels of ACE ($P < 0.001$), AGT ($P < 0.001$) and AT_1R ($P < 0.001$) gene expression between groups (Figure 7.3). Overall, cats in the natCKD had higher transcript levels of AGT and lower levels of ACE than those in control groups. The previously ischemic kidneys of the RI-DCN group tended to have mean transcript levels of all genes that were similar to those observed in natCKD kidneys. There were no significant differences in transcript levels of renin ($P = 0.543$) between groups.

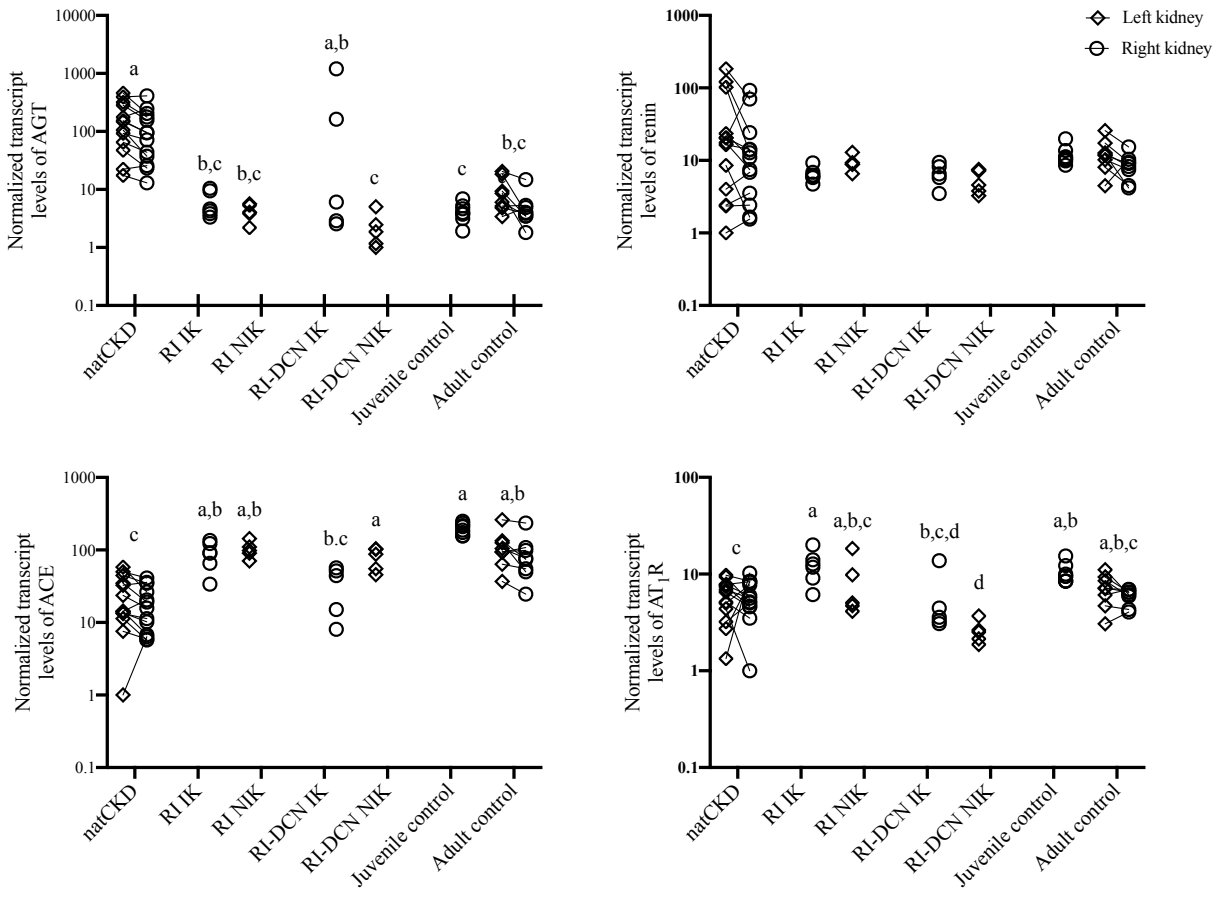

Figure 7.3. Dot plots of normalized transcript levels of angiotensinogen (AGT), renin, angiotensin-converting enzyme (ACE) and angiotensin II subtype 1 receptor (AT_1R) in renal tissue homogenates from cats with naturally-occurring chronic kidney disease (natCKD; n = 13), cats who underwent transient, unilateral renal ischemia (RI; n = 6); cats who underwent transient, unilateral renal ischemia followed by delayed contralateral nephrectomy (RI-DCN; n = 5), healthy juvenile control (n = 8) and adult control cats (n = 8). For the cats who underwent experimentally-induced unilateral renal injury (RI and RI-DCN groups, the previously ischemic kidney (NIK) and the contralateral, non-ischemic kidney (NIK) were compared separately. When bilateral samples were available and analyzed as a group, values from the left (open diamond) and right kidney (open circle) of the same individual are connected by solid lines. Levels of each target gene were normalized to those of the reference genes GAPDH and RPS7. For each gene,

transcript levels are scaled to that of the lowest sample; notice that scales differ among genes. [a,b,c,d] Groups with different letters differ significantly ($P < 0.05$).

Renal angiotensin peptides. There were significant differences in renal tissue concentrations of AngI ($P < 0.001$), AngII ($P = 0.035$) and AngIII ($P = 0.001$) between groups (Figure 7.4). Mean concentrations of AngI were the highest in kidneys from the natCKD group, when compared to all other groups. Mean concentration of AngII was numerically higher in natCKD kidneys than all other groups, and was more than double of that in adult controls (920.7 ± 739.8 fmol/g for natCKD vs. 402.8 ± 288.3 fmol/g); however, no paired comparisons were significant once corrected for multiple comparisons. For AngIII, mean concentrations were the highest in the previously-ischemic kidneys of the RI-DCN cats, though those were not significantly different from control kidneys. Concentrations of AngIV were below the level of detection and reported as < 4 fmol/g for all but two natCKD, three RI, one RI-DCN, three adult control and two juvenile control kidneys. The range of AngIV concentrations for kidneys with a value within the reportable interval for the assay were 4.6-5.0 fmol/g for natCKD kidneys, 14.9-23.5 fmol/g for non-ischemic kidneys of RI cats, 4.5 fmol/g for one non-ischemic kidney of a RI-DCN cat, 5-8.6 fmol/g for adult control kidneys, and 12.3-18.9 fmol/g for juvenile control kidneys.

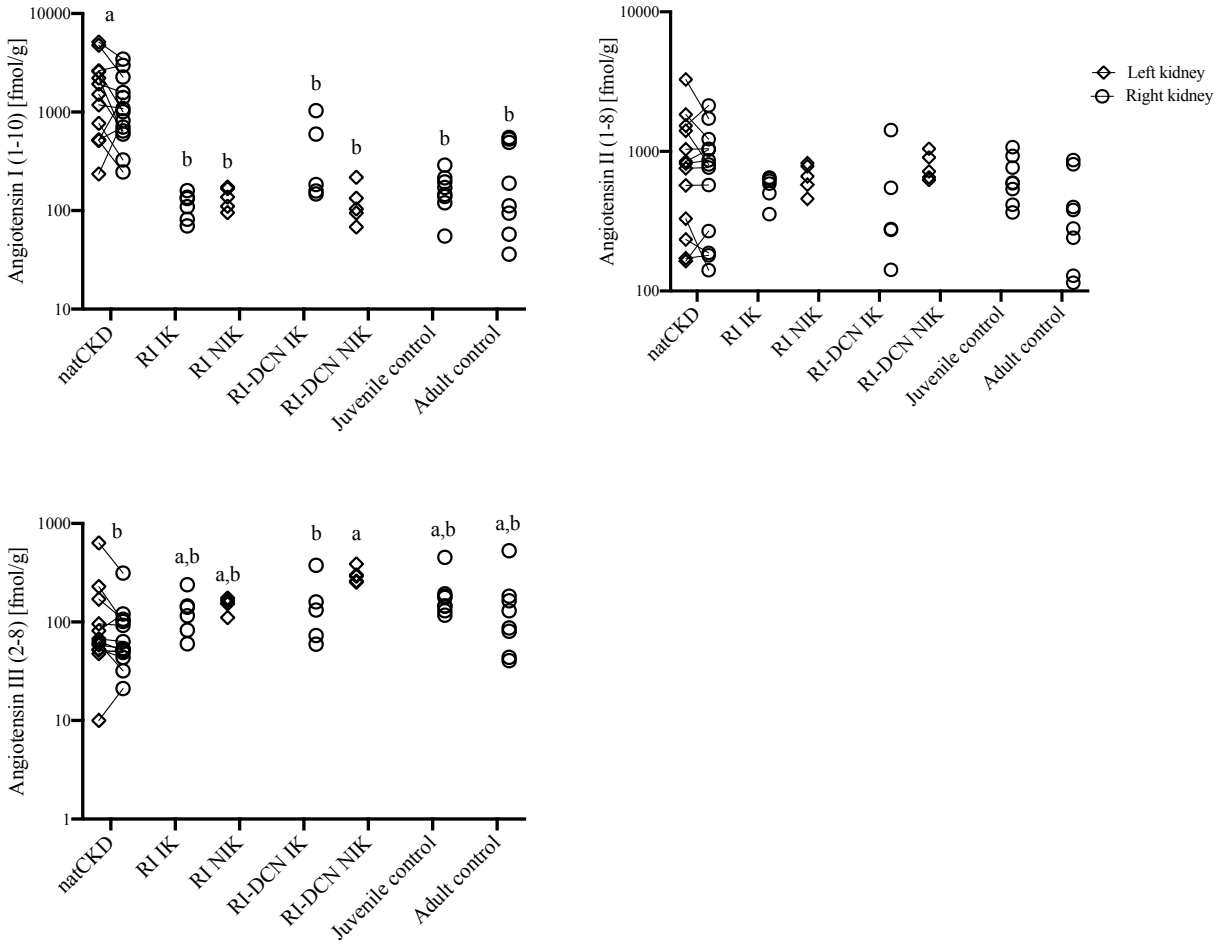

Figure 7.4. Dot plots of renal concentrations of angiotensin I (1-10), angiotensin II (1-8), and angiotensin III (2-8) in renal tissue homogenates from cats with naturally-occurring chronic kidney disease (natCKD; n = 13); cats who underwent transient, unilateral renal ischemia (RI; n = 6); cats who underwent transient, unilateral renal ischemia followed by delayed contralateral nephrectomy (RI-DCN; n = 5); healthy juvenile control cats (n = 8); and adult control cats (n = 8). For cats who underwent experimentally-induced unilateral renal injury (RI and RI-DCN groups), the previously ischemic kidney (IK) and the contralateral, non-ischemic kidney (NIK) were compared separately. When bilateral samples were available and analyzed as a group,

values from the left (open diamond) and right kidney (open circle) of the same individual are connected by solid lines. [a,b] Groups with different letters differ significantly ($P < 0.05$).

Markers of circulating RAAS. At euthanasia, there was an overall difference in mean PRA values of the experimentally-induced CKD and control cat groups ($P = 0.042$; Figure 7.5), with the mean PRA of adult control cats (3.03 ± 0.33 [ng/mL]/h) being significantly higher than the mean of the RI group cats (0.60 ± 0.17 [ng/mL]/h; $P = 0.001$), but not of that of the RI-DCN (2.24 ± 1.76 [ng/mL]/h) or the juvenile control (1.83 ± 1.11 [ng/mL]/h).

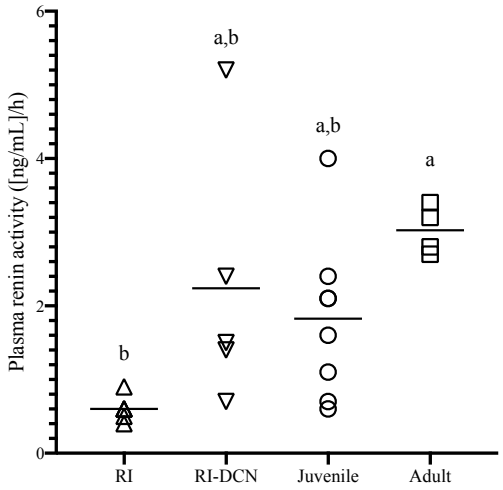

Figure 7.5. Dot plot of plasma renin activity measured in samples collected within 24 hours of euthanasia of cats subjected to transient, unilateral renal ischemia (RI group, n = 6), cats subjected to transient, unilateral renal ischemia, followed by delayed contralateral nephrectomy (RI-DCN group, n = 5), healthy juvenile control cats (n = 8) and healthy adult control cats (n = 8). Horizontal lines represent the mean for each group. [a,b] Groups with different letters differ significantly ($P < 0.05$).

There was no significant difference in mean UACR values at euthanasia between groups ($P = 0.188$; Figure 7.5), though mean values in the RI-DCN (148.0 ± 79.3) were numerically higher than those of the RI (74.4 ± 16.3), juvenile control (81.8 ± 34.0) and adult control (71.6 ± 62.1) groups.

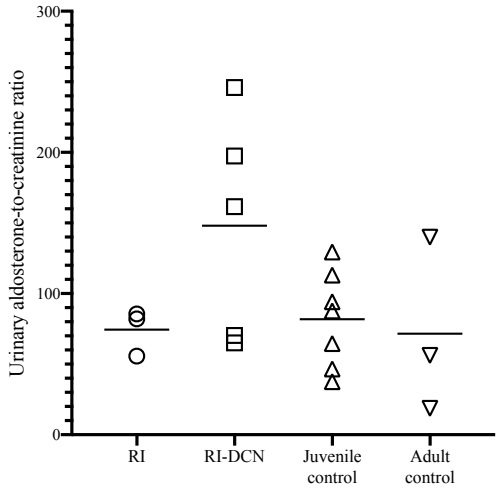

Figure 7.6. Dot plot of urinary aldosterone-to-creatinine ratio measured in samples collected within 24 hours of euthanasia of cats subjected to transient, unilateral renal ischemia (RI group, n = 3); cats subjected to transient, unilateral renal ischemia, followed by delayed contralateral nephrectomy (RI-DCN group, n = 5); healthy juvenile control cats (n = 7); and healthy adult control cats (n = 3). Horizontal lines represent the mean for each group.

When PRA measured before and after experimentally-induced renal injury were compared, in the RI group PRA significantly decreased at day 180 compared to baseline ($P < 0.001$; Figure 7.7).

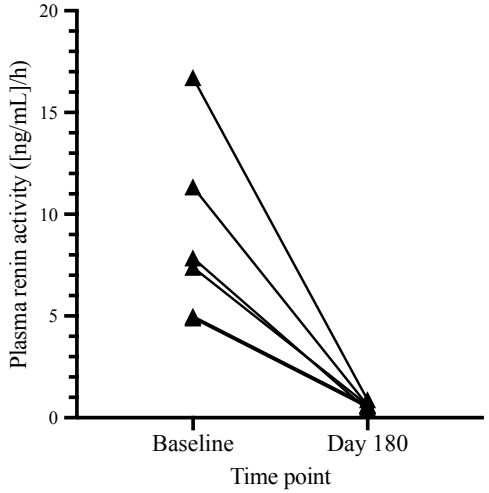

Figure 7.7. Dot plot of plasma renin activity measured five days before, and 180 days after, transient, unilateral, *in vivo*, renal ischemia (RI group; n = 6).

For the RI-DCN group, PRA was significantly increased at day 97 when compared to baseline ($P = 0.012$), but then decreased to pre-surgical, baseline levels by study end (Figure 7.8). Conversely, for UACR, there were marginally significant increases compared to baseline at day 89 ($P = 0.052$) and significant increases compared to baseline at day 269 ($P = 0.029$; Figure 7.9).

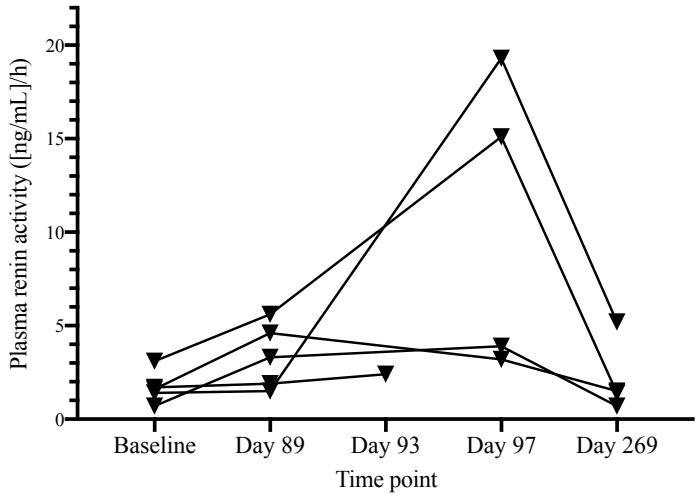

Figure 7.8. Dot plot of plasma renin activity measured before and after experimentally-induced renal injury in five cats (RI-DCN group). Baseline measurements were performed 14 days before intervention. Cats underwent unilateral renal ischemia at day 0 and contralateral nephrectomy at day 90. One cat was euthanized at day 93. All other cats were followed for 270 days post-ischemia.

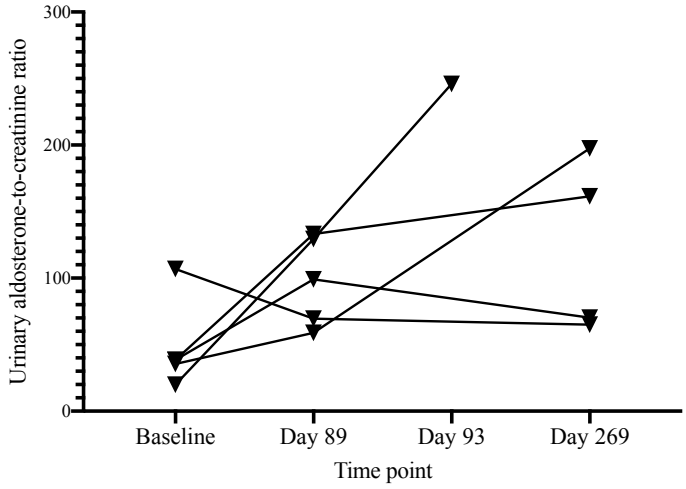

Figure 7.9. Dot plot of urinary aldosterone-to-creatinine ratio measured before and after experimentally-induced renal injury in five cats (RI-DCN group). Baseline measurements were performed 14 days before intervention. Cats underwent unilateral renal ischemia at day 0 and

contralateral nephrectomy at day 90. One cat was euthanized at day 93. All other cats were followed for 270 days post-ischemia.

Association of circulating RAAS markers with intrarenal RAS expression. There was no significant correlation between PRA or UACR and any of the renal RAS gene transcript levels or angiotensin peptide concentrations in adult controls or juvenile control cats (Table 7.5).

Table 7.5. Pearson correlation coefficients (r) and respective *P*-value for the association between plasma renin activity and urinary aldosterone-to-creatinine ratio measurements with natural log-transformed renal transcript levels of renin-angiotensin system genes and renal angiotensin peptide concentrations in healthy adult cats (n = 8) and in healthy juvenile cats (n = 8).

			Renal transcript levels				**Renal peptide concentrations**		
			lnACE	**lnAGT**	**lnAT$_1$R**	**lnRenin**	**lnAngI**	**lnAngII**	**lnAngIII**
Adult control	**PRA**	*r*	0.19	0.25	-0.59	-0.04	0.78	-0.70	-0.66
		P	0.651	0.549	0.122	0.930	0.221	0.303	0.345
		n	4	4	4	4	4	4	4
	UACR	*r*	0.03	-0.60	-0.85	-0.37	0.61	0.05	0.48
		P	0.948	0.210	0.033	0.467	0.586	0.971	0.680
		n	3	3	3	3	3	3	3
Juvenile controls	**PRA**	*r*	0.03	0.22	-0.09	0.45	0.55	0.01	-0.05
		P	0.942	0.606	0.834	0.258	0.155	0.976	0.908
		n	8	8	8	8	8	8	8
	UACR	*r*	0.22	-0.57	0.62	-0.12	0.28	0.31	-0.02
		P	0.632	0.179	0.135	0.795	0.542	0.504	0.967
		n	7	7	7	7	7	7	7

Abbreviations: ACE, angiotensin-converting enzyme; AGT, angiotensinogen; Ang, angiotensin; AT1R, angiotensin II subtype 1 receptor; PRA, plasma renin activity; UACR, urinary aldosterone-to-creatinine ratio.

Note that the number of observations was limited by sample availability and, therefore, is smaller than number of cats in the group in some instances.

In the RI group cats, for which both the previously ischemic and the non-ischemic, contralateral kidney were collected concurrently with the plasma and urine samples used for PRA and UACR, association of circulating and intrarenal markers evaluated at euthanasia was explored separately for each kidney (Table 7.6). There was no significant association between PRA or UACR and any markers of intrarenal RAS assessed in the non-ischemic kidneys from these cats. Conversely, PRA was significantly, strongly, negatively correlated with the gene transcript levels of renin in the ischemic kidneys.

Table 7.6. Pearson correlation coefficients (r) and respective *P*-value for the association between plasma renin activity and urinary aldosterone-to-creatinine ratio measurements with natural log-transformed renal transcript levels of renin-angiotensin system genes and renal angiotensin peptide concentrations measured in the non-ischemic kidneys or the previously-ischemic kidneys of cats subjected to transient unilateral renal ischemia six months prior (n = 6; RI group).

			Renal transcript levels				**Renal peptide concentrations**		
			lnACE	**lnAGT**	**lnAT$_1$R**	**lnRenin**	**lnAngI**	**lnAngII**	**lnAngIII**
NIK	**PRA**	*r*	0.54	-0.85	0.85	-0.45	-0.54	-0.46	0.14
		P	0.351	0.069	0.067	0.442	0.347	0.437	0.823
		n	5	5	5	5	5	5	5
	UACR	*r*	-0.90	0.87	-0.78	-0.50	0.76	0.22	-0.02
		P	0.289	0.329	0.430	0.664	0.448	0.856	0.990
		n	3	3	3	3	3	3	3
IK	**PRA**	*r*	-0.03	-0.24	-0.07	-0.92	-0.12	0.00	-0.74
		P	0.954	0.653	0.899	**0.011**	0.826	0.993	0.095
		n	6	6	6	6	6	6	6
	UACR	*r*	-0.39	0.64	-0.39	0.97	-0.99	-0.33	0.95
		P	0.745	0.558	0.742	0.153	0.076	0.784	0.210
		n	3	3	3	3	3	3	3

Abbreviations: ACE, angiotensin-converting enzyme; AGT, angiotensinogen; Ang, angiotensin; AT1R, angiotensin II subtype 1 receptor; IK, ischemic kidney; NIK, non-ischemic kidney; PRA, plasma renin activity; UACR, urinary aldosterone-to-creatinine ratio.

Note that the number of observations was limited by sample availability and therefore, is smaller than number of cats in the group in some instances.

In cats from the RI-DCN group, plasma and urine samples were collected the day before contralateral nephrectomy (day 89, within 24 hours of non-ischemic kidney sampling) and the day before euthanasia (day 269, within 24 hours of previously-ischemic kidney sampling), allowing for direct assessment of the association between the circulating RAAS markers and intrarenal RAS expression of each kidney at each of the two time points (i.e., nephrectomy and euthanasia). There was a significant, strong correlation between each of PRA and UACR measured at day 89 and the gene transcript levels of ACE measured in non-ischemic, contralateral kidney, which was sampled at nephrectomy at day 90 (Table 7.7). Plasma renin activity and renal ACE were positively correlated, while UACR and renal ACE were negatively correlated. At euthanasia (day 270), when only the previously ischemic kidney was present and sampled, there was a significant, strong, positive correlation between PRA measured at day 269 and each of renal transcript levels of angiotensinogen and renal concentrations AngI, AngII and AngII (Table 7.8). There was no association between UACR and any of the intrarenal RAS measurements in the previously ischemic kidneys of this CKD model.

Table 7.7. Pearson correlation coefficients (r) and respective *P*-value for the association between plasma renin activity and urinary aldosterone-to-creatinine ratio measurements with natural log-transformed renal transcript levels of renin-angiotensin system genes and renal angiotensin peptide concentrations measured in the non-ischemic kidney of cats subjected to transient unilateral renal ischemia three months prior (n = 5; RI-DCN group, samples taken at the time of delayed nephrectomy).

		Renal transcript levels				**Renal peptide concentrations**		
		lnACE	**lnAGT**	**lnAT1R**	**lnRenin**	**lnAngI**	**lnAngII**	**lnAngIII**
PRA	*r*	0.94	-0.47	0.55	0.74	-0.19	0.51	-0.03
	P	**0.019**	0.423	0.342	0.149	0.765	0.380	0.958
UACR	*r*	-0.95	0.47	-0.58	-0.76	0.12	-0.45	-0.02
	P	**0.013**	0.425	0.302	0.138	0.844	0.449	0.977

Abbreviations: ACE, angiotensin-converting enzyme; AGT, angiotensinogen; Ang, angiotensin; AT1R, angiotensin II subtype 1 receptor; PRA, plasma renin activity; UACR, urinary aldosterone-to-creatinine ratio.

Table 7.8. Pearson correlation coefficients (r) and respective *P*-value for the association between plasma renin activity and urinary aldosterone-to-creatinine ratio measurements with natural log-transformed renal transcript levels of renin-angiotensin system genes and renal angiotensin peptide concentrations measured in the previously ischemic kidney of cats (n = 5; RI-DCN group samples obtained at time of euthanasia) subjected to transient unilateral renal ischemia nine months, and contralateral nephrectomy six months, prior.

		Renal transcript levels				**Renal peptide concentrations**		
		lnACE	**lnAGT**	**lnAT1R**	**lnRenin**	**lnAngI**	**lnAngII**	**lnAngIII**
PRA	*r*	-0.62	0.93	0.07	0.68	0.92	0.97	0.95
	P	0.266	**0.022**	0.911	0.206	**0.028**	**0.005**	**0.014**
UACR	*r*	-0.66	0.47	0.57	0.44	0.60	0.51	0.28
	P	0.226	0.427	0.314	0.463	0.287	0.377	0.645

Abbreviations: ACE, angiotensin-converting enzyme; AGT, angiotensinogen; Ang, angiotensin; AT1R, angiotensin II subtype 1 receptor; PRA, plasma renin activity; UACR, urinary aldosterone-to-creatinine ratio.

When the association between the circulating RAAS and intrarenal RAS was investigated using the available information from all cats in the RI, RI-DCN, juvenile and adult control groups for which paired PRA or UACR and intrarenal RAS expression was assessed concurrently, irrespective of grouping, there was a significant, moderate, negative correlation between PRA and the renal gene transcript levels of AT_1R (Table 7.9). Plasma renin activity was also significantly, moderately, and positively correlated with renal concentrations of AngIII. The UACR was significantly, moderately, negatively correlated with gene transcript levels of ACE, and significantly, moderately, positively correlated with the renal concentrations of AngI.

Table 7.9. Pearson correlation coefficients (r) and respective *P*-value for the association between plasma renin activity and urinary aldosterone-to-creatinine ratio measurements with natural log-transformed renal transcript levels of renin-angiotensin system genes and renal angiotensin peptide concentrations in kidneys from cats with experimentally-induced chronic kidney disease (n = 11; 6 cats subjected to unilateral renal ischemia, and 5 cats subjected to unilateral renal ischemia with delayed contralateral nephrectomy), healthy adult control cats (n = 8) and healthy juvenile control cats (n = 8).

		Renal transcript levels				**Renal peptide concentrations**		
		lnACE	**lnAGT**	**lnAT1R**	**lnRenin**	**lnAngI**	**lnAngII**	**lnAngIII**
PRA	*r*	-0.11	0.22	-0.43	0.11	0.28	0.37	0.45
	P	0.551	0.232	**0.015**	0.566	0.148	0.054	**0.017**
	n	23	23	23	23	23	23	23
UACR	*r*	-0.58	0.32	-0.20	-0.16	0.61	0.11	0.13
	P	**0.002**	0.107	0.322	0.427	**0.002**	0.609	0.546
	n	18	18	18	18	18	18	18

Abbreviations: ACE, angiotensin-converting enzyme; AGT, angiotensinogen; Ang, angiotensin; AT1R, angiotensin II subtype 1 receptor; PRA, plasma renin activity; UACR, urinary aldosterone-to-creatinine ratio.

Note that the number of observations was limited by sample availability and, therefore, smaller than the total number of cats in the study.

For the renal ischemia group cats, PRA and UACR were assessed against renal transcript levels and peptide concentrations in the previously-ischemic kidneys.

Discussion

The present study showed that kidneys from cats with naturally-occurring CKD demonstrated higher AGT, and lower ACE, transcript levels than those of healthy control cats. In

the same cats, mean AngI (i.e., angiotensin [1-10]) concentrations were significantly higher than in cats with experimentally-induced CKD and in healthy control cats. Mean concentrations of AngII (i.e, angiotensin [1-8]) were numerically higher in the kidneys of natCKD cats; however, while there was a significant difference in concentrations of this peptide among all kidney groups, variances of the data were large and no pairwise comparisons were significantly different when adjusted for multiple comparisons.

Recently, local, tissular RASs have gained attention as crucial mediators of end-organ damage.[45] Importantly, these local systems appeared to be subject to mechanisms of regulation that are independent of the classical, circulating RAAS.[41, 42, 46, 49] In circulation, concentrations of angiotensinogen are abundant, with renin activity representing the rate-limiting, major regulatory step of the RAAS.[33, 34] Conversely, in the kidney, generation of AngII is controlled by numerous independent mechanisms, of which intrarenal generation of AGT appears to be a critical factor.[49] Our data would suggest that, based on the abundance of AGT mRNA, the intrarenal RAS is activated in feline CKD. Interestingly, ACE mRNA levels appeared scarce in tissues from affected cats, when compared to those from healthy individuals. Additionally, while AngI levels were significantly higher and Ang II levels were numerically greater in natCKD tissues, AngII was not significantly different for any direct group comparisons. Because the significance levels for these evaluations were adjusted to minimize type II error rate associated with multiple comparisons, it remains possible that AngII concentrations are, in fact, higher in feline CKD than in health, and present a substantial contributor to ongoing renal damage. If that is the case, a significant difference might have been observed if this study was designed to solely compare natCKD and the control groups and/or had included a larger number of cats, thereby increasing the power to detect a difference. An argument could be made that the noted abundance in AngI

with an apparent lack of AngII increase in CKD could reflect the relative lack of ACE, the enzyme responsible for forming AngII from AngI; however, ACE-independent pathways, which allow for production of AngII while bypassing ACE activity, appear to predominate in renal tissues.[42] One of such pathways relies on the activity of the enzyme chymase,[151] which was not evaluated in the present study.

Overall, mean PRA and UACR at the time of euthanasia in the four cat groups for which these parameters were measured were within the ranges reported for healthy individuals in previous studies.[211, 212, 313, 314] In experimentally-induced CKD, as in previous studies,[207, 208] it appears that differences in PRA and UACR between affected and control cats differ according to the specific model. In the models evaluated here, 90-minutes of *in vivo*, unilateral renal ischemia alone appeared to have resulted in a downregulation of the circulating RAAS, as PRA was significantly lower in the RI group cats than in adult controls, although UACR was not different among groups. Additionally, in that model, PRA was significantly lower six months after renal ischemia when compared to baseline. Importantly, cats subjected to transient renal ischemia as the sole intervention were intact and juvenile (medium [range] age, 329 [281-397] days) at baseline (5 days before renal injury). At day 0 of the original study, the cats underwent ovariohysterectomy, alongside the intended renal ischemia. By the time of euthanasia, all cats had therefore entered adulthood (median [range] age, 520 [472-588] days) and had been sexually altered for six months. In human beings, both gender and age are known factors in the regulations of RAAS,[315-318] with young children having higher supine PRA and plasma aldosterone than adolescents.[318, 319] Similarly, a feline study documented an association between aging and a decline in PRA in healthy individuals.[313] However, plasma aldosterone concentration remained unchanged with increasing age in the same cats, leading to an increase in the

aldosterone-to-renin ratio in older cats. To address the potential impact of age in our study, we chose to include both a juvenile control group comprised of young cats, as well as an adult control group which included skeletally mature animals. While subtle differences were observed in pairwise comparisons of intrarenal RAS expression between the two control groups, significant differences in PRA and UACR were not observed; however, our samples sizes were relatively small and the cats in our adult control group were still of a young age. Javadi and colleagues documented a significant increase in aldosterone-to-renin ratio for cats older than 5 years of age,[313] an age that was not reached by the majority of the cats in our adult group. Additionally, in the present study, gender distribution differed between juvenile and adult controls. In human beings, differences in circulating and/or urinary renin and aldosterone concentrations among men, pre- and post-menopausal women, and women receiving estrogen replacement therapy, have been documented in multiple studies,[320-322] although evidence is occasionally conflicting in regards to the directionality of the observed disparities.[316] In cats, neutering has been associated with lower PRA, though not with a change in plasma aldosterone concentration.[313] It is, therefore, possible that competing factors may have influenced our results.

In the RI-DCN cats, a significant increase in PRA was observed at day 97 (i.e., 97 days after unilateral renal ischemia and seven days after nephrectomy of the contralateral, non-ischemic kidney), though PRA later returned to baseline levels. Apparent activation of the circulating RAAS, as indicated by PRA, may have reflected an initial adaptive change allowing for maintenance of GFR and normal circulating blood volume following ischemia- and nephrectomy-induced functional renal mass reduction. Theoretically, the need for RAAS activation may have reduced over time once morphologic changes, such as glomerular hypertrophy, occurred. Of note is the fact that UACR remained elevated at study completion,

when compared to values before intervention. Therefore, despite the renin activity having returned to baseline, RAAS stimulation appeared sustained when aldosterone was considered, an observation that resembles low-renin hypertension in human beings,[323, 324] as well as naturally-occurring feline hypertension.[209, 211, 212]

Data from the RI-DCN, which allow for evaluation of earlier time points post-renal injury also raises the question of whether a transient increase in PRA may have been missed in the RI group, as no evaluation was performed prior to day 180. In rats subjected to 60 minutes of unilateral renal ischemia, urinary concentrations of AngI and angiotensin (1-7) significantly increased at 24 hours post-ischemia, but declined to control levels by 72 hours.[325] If a similar pattern was present in the RI group, it would have been overlooked.

A secondary objective of the present study was to evaluate the correlation between markers of the circulating RAS and expression of intrarenal RAS components. When PRA and UACR were assessed for possible association with expression of the intrarenal RAS components, the results varied substantially among groups. In cats of the RI-DCN group, PRA was positively correlated with renal ACE transcript levels of the non-ischemic kidney, while UACR and renal ACE were negatively correlated. At the time of collection of the non-ischemic kidney, the previously ischemic kidney was not sampled and remained untouched *in vivo*, which may have substantially impacted these results if each kidney was contributing to the RAAS differently. At euthanasia and final sample collection for these cats, only the previously ischemic kidney was present. At that time point, PRA was associated with each of renal transcript levels of angiotensinogen and renal concentrations AngI, AngII and AngII. In the RI group, there were significant associations between PRA and intrarenal RAS expression in the previously ischemic kidneys, but not the non-ischemic kidneys, further suggesting that the presence of the previously

ischemic kidney at day 90 in the RI-DCN poses a confounding factor for our results. In healthy control cats, there was no significant association between PRA or UACR and either of the measures on intrarenal RAS expression here studied. These data suggest that a consistent estimation of intrarenal RAS activation cannot be made using PRA or UACR, and there is need for the identification of a biomarker of the local RAS that would allow for evaluation of this system in a less invasive manner. Studies in human beings suggest that urinary angiotensinogen is suitable biomarker for assessment of intrarenal AngII,[326, 327] warranting future investigation biomarker of intrarenal RAS activity in cats.

There are several limitations to the present study. Firstly, we were limited by the number of cats for which banked samples were available, which alongside the high variability and wide ranges of the data here presented, may have substantially impacted our power to detect differences between groups. This issue was also compounded by the study design, which included multiple comparisons, and further promoted a higher likelihood of a type I error. Another limitation is the lack of SBP data for cats with natCKD or healthy cats. In the two CKD models, SBP measurements were collected over the course of each respective study. A future study should aim to include SBP in the statistical models. Moreover, due to the origin of our samples, dietary sodium intake was not standardized for all groups, and may have, in part, impacted our observations. While we had no information regarding the diet of four of the adult control cats, it was known that cats in the RI and juvenile control groups, as well as half of the cats in the adult control group were being fed a relatively high sodium diet when compared to most cats in the natCKD and RI-DCN groups. Further, cats in the RI-DCN group had a substantial reduction in sodium intake during the course of the original model study, which may have impacted their results, as their diet at baseline differed from that of all other time points.

Additionally, as blood and urine collection had not been performed for client-owned cats, no evaluation of the circulating RAAS was performed for natCKD, leaving an important gap in the assessment of this system in that group. Finally, no assessment of ACE-independent pathways was attempted, which could have benefited the interpretation of our data. A future direction for the investigation here started may include a differently designed study, with simpler and fewer comparisons, and ideally a larger number of individuals for which SBP and dietary data is known. Further, a direct analysis of objective measures of GFR and the RAAS may also provide relevant information.

In conclusion, when considering a portion, though not all of the data here described, the intrarenal RAS appears to be overstimulated in both naturally occurring and a unilateral ischemia-delayed contralateral nephrectomy CKD model in cats. As in other species,[215, 328] markers of circulating RAAS activity appear to be poor and inconsistent predictors of intrarenal RAS activity in the models of feline CKD here studied and in healthy control cats. Assessment of RAAS activity requires a comprehensive evaluation of its circulating and intrarenal components. Future efforts towards identifying biomarkers reflecting the status of the intrarenal RAS are vital for *in vivo* research in clinical patients.

Footnotes

a. Attoquant Diagnostics, Vienna, Austria.

b. Ethylenediaminetetraacetic acid, Sigma-Aldrich, Steinheim, Germany.

c. Z-Pro-Prolinal, Sigma-Aldrich, Steinheim, Germany.

d. 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride, Sigma-Aldrich, Steinheim, Germany.

e. Aminopeptidase inhibitor, Sigma-Aldrich, Steinheim, Germany.

f. Dulbecco's PBS, Sigma-Aldrich, Steinheim, Germany.

g. Xevo TQ-S triple quadruple mass spectrometer, Waters Corporation, Milford, MA.

h. Michigan State University Veterinary Diagnostic Laboratory, Lansing, MI.

i. Active Aldosterone RIA, DSL8600, Beckman Coulter, Fullerton, CA.

j. RNA*later* stabilization solution, QIAGEN, Valencia, CA.

k. RNeasy Plus Mini Kit, QIAGEN, Valencia, CA.

l. NanoDrop Spectrophotometer, Thermo Fisher Scientific, Waltham, MA.

m. ezDNase, Invitrogen, Carlsbad, CA.

n. SuperScript IV VILO Master Mix, Invitrogen, Carlsbad, CA.

o. CFX96, Bio-Rad Laboratories, Hercules, CA.

p. SsoAdvanced SYBR Green Supermix, Bio-Rad Laboratories, Hercules, CA.

q. PGEM-T easy vector, Promega, Madison, WI.

r. Molecular Cloning Laboratories, San Francisco, CA.

s. Guanidinium chloride, Sigma-Aldrich, Steinheim, Germany.

t. Trifluoroacetic acid, Sigma-Aldrich, Steinheim, Germany.

u. 2mm microtip, Sonics and Materials, Newton, NJ.

v. SAS V 9.4, Cary, NC.

w. GraphPad Prism for Mac, version 7, GraphPad Software Inc, La Jolla, CA.

CHAPTER 8

SUMMARY AND CONCLUSIONS

Chronic kidney disease, a prevalent metabolic disorder in aged companion animals, negatively impacts the quality of life and life span of affected individuals.[3, 11] Proteinuria, chronic hypoxia and activation of the RAAS are known contributors to progressive renal damage in CKD.[15, 16] As the progression of CKD is multifactorial, treatment aimed at halting it should likely be multimodal. While therapeutic strategies targeting proteinuria and overactivity of the RAAS are available, these are not uniformly effective.[143, 329] Additionally, therapies directly targeting pro-inflammatory and pro-fibrotic pathways activated by chronic renal hypoxia are not currently available. To address these gaps, the work described here aimed to identify the superior of two drugs used for the treatment for canine proteinuric CKD, and to characterize molecular pathways associated with renal hypoxia, as well as the intrarenal RAS, in feline CKD.

Glomerular disease is common in dogs. Therefore, proteinuric CKD is frequently observed in this species. Proteinuria is a predictor of renal morbidity and mortality, as well as all-cause mortality, in canine CKD,[140-142] and antiproteinuric treatment is associated with decreased progression of CKD.[143-145] Angiotensin-converting enzyme inhibition is a mainstay of the standard treatment canine renal proteinuria;[147] however, a subset of patients will fail to respond to therapy.[143] Pre-clinical data suggest that the ARB telmisartan achieves superior RAAS blockade to that of the ACEi enalapril.[162] Therefore, we hypothesized that telmisartan would be a superior antiproteinuric agent in dogs. In the first manuscript, presented as the third chapter of the present dissertation, we describe the results of a prospective, randomized, double-masked,

clinical trial comparing the antiproteinuric effects of telmisartan with those of enalapril in dogs with CKD. Over a 90-day period, at the dosages and up-titration schedule used, telmisartan achieved greater median proteinuria reduction, as well as clinically-relevant reduction (defined as ≥ 50% decrease in UPC as compared to baseline), in a shorter period of time. At day 90, when dogs with persistent proteinuria were prescribed a combination of the two RAAS blockers, further reduction in UPC was observed; however, this practice was associated with substantial risk for azotemia. Our data support that, as in human beings,[159, 241, 242] combination of RAAS blockers may be associated with increased risk for adverse events and should be prescribed with caution. When prescribed alone, no apparent differences in the safety profile of these medications were observed. Thus, as the efficacy of telmisartan was greater than that of enalapril, our results suggest the former as a candidate for first-line therapy in dogs with proteinuric CKD. Long-term follow-up studies are needed to assess the impact of telmisartan in progression of CKD and renal survival in this species.

In cats, CKD is typically characterized by primary lesions of the tubulointerstitial compartment.[182, 183] Although proteinuria is a strong predictor of survival in feline CKD,[219, 222] glomerular disease and heavy proteinuria are uncommon in domestic felids.[302] Hypoxia, along with aging, appear to be crucial progression factors for feline CKD.[182] In culture cells and rodent models, hypoxia has been shown to trigger the expression of several pro-fibrotic and pro-inflammatory cytokines.[24, 91] Such molecular pathways induced by renal hypoxia are poorly characterized in the cat. To describe the alterations in gene transcription following hypoxic events, we first evaluated renal tissues from cats who underwent transient, *in vivo*, unilateral renal ischemia six months prior as a model of renal fibrosis and CKD. In the study reported in the second manuscript (fourth chapter) of this dissertation, we used RT-qPCR to measure

transcript levels of specific fibrosis mediators, each having been previously described in detail in non-feline CKD. In this model, we documented upregulation of transcription of MMP-2, -7 and -9, TIMP-1, and TGF- β1, as well as a downregulation of transcription of VEGF-A. Transcription of HIF-1α was not different among groups. Importantly, differential regulation of TIMP-1 and VEGF-A transcription was observed in the contralateral, non-ischemic kidneys, when compared to control kidneys, suggesting that mechanisms of crosstalk *in vivo* influence the transcriptome in organs not directly affected by hypoxia. In kidneys previously subjected to ischemic injury, transcript levels of HIF-1α, MMP-2, MMP-7, and TIMP-1 were positively correlated with worse degrees of fibrosis, presenting opportunities for development of biomarkers of renal fibrosis.

To characterize other potentially relevant pathways not targeted by our RT-qPCR study, we then used RNA sequencing to produce and analyze whole-renal transcriptome reads. Using this technique, we observed differential transcription of thousands of genes and identified additional disease markers, such as fibulin-1, secreted phosphoprotein-1 and matrix Gla protein. Once again, we documented not only that an alteration in gene transcription was present six months after injury, but that unilateral renal injury influenced gene transcription in both kidneys.

With the aim of assessing whether the above described hypoxia-induced pathways are similarly transcribed in naturally-occurring CKD, we prospectively recruited tissues from affected animals. We collected renal samples from 13 cats diagnosed with various stages of CKD. As in our tissues from the disease models, we noted increased transcript levels of MMP-2, -7 and -9, TIMP-1, and TGF- β1, with concurrently decreased levels of VEGF-A transcripts in diseased kidneys, when compared with controls. Transcripts of HIF-1α were also significantly increased in tissues from cats with spontaneous CKD. In the CKD tissues, transcript levels of

HIF-1α, MMP2, MMP-7 and TGF-β1 were positively correlated with worse histologic lesion scores, supporting the role of hypoxia and these specific molecular pathways in the characteristic fibrogenesis of feline CKD.

Finally, the role of the RAAS in feline CKD is a subject of debate. Mixed evidence has been presented by different studies of experimentally-induced[207, 208] and spontaneous CKD.[209, 211, 212] The intrarenal RAS has been increasingly recognized as a contributor to the pathogenesis of renal disease and hypertension in other species.[330, 331] Nonetheless, most feline studies to-date have focused on the systemic, circulating RAAS, by evaluating PRA or plasma aldosterone concentration. Data obtained from rodent models reveal that regulation of the intrarenal RAS is independent of that of its circulating counterpart.[49] Therefore, characterization of this local system is critical for the evaluation of RAAS in feline CKD. In the last manuscript here included, we sought to describe the intrarenal RAS in feline CKD, and to assess the association between markers of the circulating RAAS and those of the intrarenal system in cats. Using banked samples, we measured transcript levels of four main RAS components (i.e., AGT, renin, ACE and AT_1R) and renal angiotensin peptides in samples from cats with spontaneous CKD, from two renal ischemia models, and from healthy individuals. Additionally, we measured PRA and UACR, as markers of circulating RAAS activation, in samples from the CKD models and healthy cats. Our data have shown that tissues from cats with renal disease exhibited significantly higher renal transcript levels of AGT and renal AngI concentrations than did those of healthy cats. Measurements of PRA and UACR were inconsistently correlated with intrarenal expression of the RAS. Collectively, these data support that intrarenal RAS activation may be present in feline CKD, and that may not be detected upon evaluation of PRA or UACR.

In summary, the data here reported identifies a superior antiproteinuric agent for dogs with CKD, describes specific pathways associated with the development of tubulointerstitial fibrosis in cats, and documents an upregulation of certain markers of the intrarenal RAS in feline CKD. While these data undoubtedly contribute to our knowledge of progressive CKD, many questions remain unanswered. To address the questions raised by the present work, future studies should aim to evaluate the long-term effects of telmisartan on the progression of azotemia and the survival of dogs with proteinuric CKD. For feline CKD, characterization of the protein levels of the genes here described is warranted for scrutiny of their value as biomarkers of renal fibrosis. Finally, as the RAAS is an intricate system, crucial components remain unstudied. Relevant knowledge gaps begging to be filled include the characterization of the role of ACE-independent pathways in the intrarenal generation of AngII, and the identification of a biomarker of intrarenal RAS activation, allowing for evaluation of this system in clinical patients.

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