DISSERTATION

# FELINE CHRONIC KIDNEY DISEASE: NOVEL APPROACHES TO ETIOLOGY, SPECIFIC THERAPY AND SUPPORTIVE CARE

Submitted by

Jessica M. Quimby

Department of Clinical Sciences

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2012

Doctoral Committee:

Co-Advisor: Steve Dow
Co-Advisor: Michael Lappin

Katharine Lunn
Christine Olver

ABSTRACT

FELINE CHRONIC KIDNEY DISEASE: NOVEL APPROACHES TO ETIOLOGY, SPECIFIC THERAPY AND SUPPORTIVE CARE

Chronic kidney disease is one of the leading causes of morbidity and mortality in geriatric cats, affecting conservatively 30% of the population; an estimated 24 million cats nationwide in the United States. Despite the common nature of the disease, its etiology is yet unknown, and there is no definitive cure short of renal transplantation. The goals of the research described in this dissertation were to explore possible etiologies of chronic kidney disease and to develop novel treatment strategies to help cats afflicted with this disease.

The first part of this project investigated a possible etiology for CKD; renal aging as manifested by telomere shortening and cellular senescence. In these studies telomere length and cellular senescence were assessed in cats with CKD in comparison to young healthy and geriatric healthy controls. Using a TELI-FISH assay to measure telomere length in specific renal cell populations, significantly shorter telomeres were found in the renal proximal and distal tubular cell population of CKD cats compared to young normal or geriatric normal cats. There was no difference between CKD cats and normal cats when liver or skin telomere length was measured. Additionally, β-galactosidase assay revealed increased cellular senescence in the kidneys of CKD cats in comparison to young normal. CKD cats tended to have increased β-galactosidase staining in comparison to normal geriatric cats, but this did not reach statistical significance. Neither telomere length nor cellular senescence were correlated with age, but the normal geriatric population available for assessment was small. It was concluded that telomere shortening and cellular senescence are present in feline CKD; future studies will be necessary to determine cause and effect aspects of this relationship. Demonstration of an association between telomere shortening, cellular senescence and feline CKD could be the foundation of new treatment strategies.

Cats with CKD frequently have poor appetites and nutritional management of these patients is important. Mirtazapine is an appetite stimulant and anti-nausea medication that has recently gained popularity in veterinary medicine and anecdotally appears to be helpful for the management of appetite. However, no pharmacokinetic or pharmacodynamic information exists on the drug in cats. The aims of the second part of these studies were a) the assessment of the pharmacokinetics and pharmacodynamics of commonly prescribed doses of mirtazapine in normal cats, elderly cats and cats with CKD, and b) a placebo-controlled blinded crossover clinical trial to assess the efficacy of mirtazapine in CKD cats. These studies demonstrated that there are differences in the metabolism of mirtazapine between young normal cats, geriatric normal cats and CKD cats. Based on the pharmacokinetic studies, young cats could receive daily mirtazapine at a low dose without significant likelihood of drug accumulation whereas CKD cats should receive the drug every other day due to delayed clearance. In a subsequent clinical trial, mirtazapine significantly increased appetite, activity and weight in CKD cats when administered at a low dose every other day for three weeks. Additionally, a significant decrease in vomiting was noted. This demonstrated that mirtazapine does have significant appetite stimulating and anti-nausea effects in CKD cats. The information gathered in this body of work will help clinicians prescribe mirtazapine more effectively with a decreased incidence of unwanted drug side effects. Most importantly, it will help improve the quality of life and potentially prognosis of cats suffering from CKD.

Most treatments for CKD are palliative in nature and do not directly address the underlying pathology. CKD is characterized by tubulointerstitial inflammation, fibrosis and progressive loss of renal function. Mesenchymal stem cell (MSC) therapy is thought to be anti-inflammatory, and has the potential to improve or stabilize renal function in animals with renal failure, based on evidence from rodent model studies of induced renal disease. At present, there is little published work regarding the use of MSC for treatment of naturally occurring CKD. The last section of this body of work focuses on the evaluation of MSC therapy as a novel treatment strategy for cats with CKD. A series of pilot studies was performed; a pilot study of intrarenal injection of autologous stem cells and two pilot studies of intravenously injected allogeneic cryopreserved MSC. Urinary cytokines were measured to assess intra-renal inflammation,

fibrosis and vascular health and the possible effects of MSC injection on these factors. We determined that MSC could be successfully harvested and cultured from bone marrow and adipose sources, but the latter was preferred for ease of collection, expansion and superior yield. Intrarenal injection did not induce immediate or longer-term adverse effects. Two CKD cats that received intrarenal adipose-derived MSC experienced modest improvement in GFR and a mild decrease in serum creatinine concentration. In the allogeneic cryopreserved intravenous study, six cats received $2 \times 10^6$ MSC per injection and experienced a significant decrease in serum creatinine with negligible side effects. Five cats received $4 \times 10^6$ MSC per injection and side effects included vomiting during infusion and increased respiratory rate. Variable decreases in serum creatinine, increases in GFR by iohexol clearance and changes in urinary cytokines were seen. Despite the mild improvement in creatinine seen in some of the cats, none had improvement to the extent described in rodent models. While MSC therapy potentially holds promise for palliation of CKD, additional work is necessary to determine if this therapy can be manipulated to increase its efficacy.

The work described in this dissertation has increased our knowledge of the biology of renal aging and its relationship to CKD. In addition it has assessed the effect of two novel treatment strategies on cats with CKD. This information will directly improve the lives of cats with CKD as well as providing a strong foundation for further research in this area.

# ACKNOWLEDGEMENTS

This body of work would not have been possible without the pivotal assistance of several people during my graduate career. First and foremost I would like to thank my co-advisor team, Dr. Steve Dow and Dr. Mike Lappin for their continued support and guidance through my residency and graduate program. Without them, I would not have been offered as many opportunities to achieve what I have during my program. I would also like to thank Dr. Kathy Lunn for her invaluable support, advice and friendship and for being a role model as a superb clinical scientist. I would like to acknowledge Dr. Christine Olver for her vital participation as my fourth committee member. Sincere thanks to Amber Caress for unending technical support and kitty wrangling; Dr. Shannon McLeland for being my own personal nephropathologist and always being excited about kidneys; and Dr. Tracy Webb for her experience, advice and invaluable lab expertise. The telomere project would not have been possible without the collaboration and expertise of David Maranon as well as the resources provided by Dr. Susan Bailey and Dr. Joel Bedford. The mirtazapine project would not have been possible without the support of Dr. Dan Gustafson and the folks in the Pharmacology Core. I would also like to acknowledge the valuable contributions of Lauren Habenicht and Willy Brock; their time and effort made these projects possible and so much easier. Particular thanks to Brad Charles for unending support with all things IHC. Special thanks to Tony Harp for singlehandedly participating in more clinical trials than anyone else and, in an unexpected twist of fate, for love, support and understanding in the eleventh hour. And last but certainly not least, I would like to acknowledge the many kitties who made this work possible, namely Chloe, Gus, Colby, Gretchen, Chet and Zoe. Most of them are no longer with us as they have already succumbed to this devastating disease. They will be missed and we gratefully acknowledge their contributions to make a difference for other kitties in the future.

TABLE OF CONTENTS

LIST OF TABLES

**Chapter 4**



**Chapter 5**

LIST OF FIGURES

**Chapter 1**



**Chapter 3**



**Chapter 4**

**Chapter 5**

LIST OF PERTINENT PUBLICATIONS

In vitro comparison of feline bone-marrow-derived and adipose tissue-derived mesenchymal stem cells. T.L. Webb, J.M. Quimby, S.W. Dow. *J Fel Med Surg* 2011; 14: 165-168.

The pharmacokinetics of mirtazapine in cats with chronic kidney disease and in age-matched control cats. J.M. Quimby, D.L. Gustafson, K.F. Lunn. *J Vet Int Med* 2011; 25: 985-988.

Evaluation of intrarenal mesenchymal stem cell injection for treatment of chronic kidney disease in cats: a pilot study. J.M.Quimby, T.L. Webb, D. Gibbons, S.W. Dow. *J Fel Med Surg* 2011; 13(6):418-26.

Studies on the pharmacokinetics and pharmacodynamics of mirtazapine in healthy young cats. J.M. Quimby, D.L. Gustafson, B.J. Samber, K.F. Lunn. *J Vet Pharmacol Ther* 2010: 34: 388-396.

# CHAPTER 1: LITERATURE REVIEW

## 1.1 Pathophysiology of Chronic Renal Disease

### *1.1.1 Prevalence of Chronic Kidney Disease*

Chronic kidney disease is one of the leading causes of morbidity and mortality in geriatric cats, affecting conservatively 30% of the population; an estimated 24 million cats nationwide in the United States.[1] Despite the common nature of the disease, its etiology is yet unknown. Short of renal transplant, no prevention or cure for this condition has been discovered and medical therapy for metabolic complications and alleviation of symptoms is standard of care in human and veterinary medicine.[2] This condition is characterized by tubulointerstitial damage, fibrosis and progressive loss of renal function, and is commonly described as the final common pathway after any one of multiple types of renal insults. Regardless of the initial insult, once a threshold of renal damage has been reached, progression is irreversible and appears consistent in character.

### *1.1.2 Progression of CKD*

Impairment of renal function is correlated with the degree of tubulointerstitial injury, including tubular atrophy, atubular glomeruli and tubulointerstitial fibrosis.[3] In a recent retrospective study that assessed the correlation between CKD and histopathology in cats, fibrosis was found to be significantly correlated with stage of CKD. (McLeland, unpublished data) Hyperfiltration, proteinuria, tubulointerstitial inflammation, oxidative damage, hypoxia and induction of the renin-angiotensin-aldosterone system (RAAS) are major factors that are thought to contribute to the process of tubulointerstitial injury.[4-6]

Loss of functional nephrons results in hyperfiltration due to an increase in remaining single nephron GFR. Combined with a loss of the ability to autoregulate blood flow to the kidney, hyperfiltration results in intraglomerular hypertension, which can be further exacerbated by systemic hypertension.[5] Mechanical and sheer stress on the glomerular apparatus causes damage resulting in

stretching of the membrane and leakage of proteins into the interstitium. Although the kidney filters a large amount of protein on a daily basis, urinary protein levels probably underestimate the filtered protein load due to the enormous capacity of the proximal tubule for protein reabsorption.[5] However, proximal tubule cells are therefore prone to damage as a result. Proteins are reabsorbed through megalin-cubulin mediated endocytosis and then processed lysosomally and secreted back into circulation.[5] Protein excess results in overload of the lysosomal system and the resultant rupture of lysosomes is damaging to tubular cells and instigates recruitment of inflammatory cells to the area. In addition, substances carried by the proteins such as LPS, FFA, PG, heavy metals and hormones result in oxidative stress as well as direct damage to the cells.[5]

Tubulointerstitial inflammation is characterized by an inflammatory infiltrate in the interstitium that usually consists of mononuclear leucocytes including nephritogenic T-cells, monocytes and macrophages. These cells are thought to play a pivotal role in progression and fibrosis.[7] Tubulointerstitial inflammation is more strongly correlated with decreasing renal function than glomerular damage is, even though most renal disease in humans is glomerular in origin.[3] The presence of interstitial inflammation affects the ability of the kidney to autoregulate by directly affecting the afferent arteriole's ability to respond to contractile stimuli.[8] Impaired autoregulation exacerbates the already present hyperfiltration and intraglomerular hypertension leading to additional damage. Nephritogenic T-cells are thought to exacerbate the conditions present in the kidney through direct cytotoxic effects as well as non-cytotoxic mechanisms such as cytokine release, altered tubular function and proliferation of interstitial fibroblasts and fibrosis.[5] Once fibrosis occurs, conditions become increasingly suboptimal as loss of peritubular capillaries and increased interstitial volume create resistance to oxygen diffusion and resultant hypoxia, which in turn stimulates more fibrosis. Loss of peritubular capillaries, increased interstitial volume and fibrosis are also strongly correlated with decreasing renal function as well as a worse prognosis.[3,7]

Oxidative damage to tubular cells is also an important factor in cell attrition and occurs from several sources. As previously discussed, it can occur as a result of direct harm from reabsorbed substances, some of which are attached to reabsorbed proteins. However, increased filtration of

substances such as glucose or protein also greatly increases the metabolism of tubular cells and this leads to increased production of free radicals. It has been shown that anti-oxidant defense systems are decreased in CKD, and thus the kidney is little prepared to address this increase and oxidative stress results.[9] In addition, increase metabolic demand results in hypoxia as cells receive the same amount of blood flow, but have a higher demand. Angiotensin II is upregulated in CKD and this also promotes oxidative stress by stimulating NADPH oxidase.[4] The environment is further exacerbated if anemia is present. Hypoxia results from decreased oxygen presentation to the tissues and oxidative stress is increased as erythrocytes have a major antioxidant role.[4]

The RAAS system is also a critical component in the pathophysiology of renal disease progression.[6] Although normally protective in emergency situations such as shock or hypotension, the RAAS system becomes maladaptive in CKD. Combined with a loss of autoregulation in the diseased state, angiotensin II plays a major role in this pathologic process as it vasoconstricts the efferent arteriole to maintain GFR. Although GFR is maintained, the downsides of this mechanism are hypoperfusion of post-glomerular capillaries, including the interstitium, and glomerular capillary hypertension. Angiotensin II also has detrimental non-vascular effects including activating tubular cells, oxidative stress, stimulation of inflammatory cell accumulation and promotion of fibrosis. The importance of the RAAS system is further supported by clinical evidence in humans demonstrating a distinct clinical benefit from RAAS blockade.[6]

One prevalent theme in the previous discussion that should be reiterated is the importance of hypoxia in the progression of CKD.[4] In summary, tubulointerstitial inflammation leads to loss of peritublar capillaries which decreases blood flow to the tubules and creates a hypoxic environment. Vascular endothelial growth factor (VEGF) is integral to vascular health, and it appears that late stage renal patients have a compromised VEGF levels.[10] Distortion and destruction of peritubular capillary blood supply by inflammatory infiltrates, extracellular matrix and fibrosis is a characteristic histologic feature of CKD in all species; this expansion of the interstitial area further impairs oxygen diffusion. Glomerular damage and vasoconstriction of afferent arterioles decrease postglomerular peritubular

capillary blood flow.[4] Angiotensin II also directly constricts efferent arterioles as well as induces oxidative stress which hampered efficient utilization of oxygen by tubular cells. Relative hypoxia occurs as a result of increased metabolic demand of tubular cells and anemia further exacerbates all by hindering oxygen delivery.[4] Unfortunately hypoxia is a strong stimulus for further formation of fibrosis, which sets in motion a vicious cycle of fibrosis and further hypoxia. Hypoxia also leads to apoptosis or epithelial-mesenchymal transdifferentiation of tubular cells into myofibroblasts. As a result of the multitude of factors involving hypoxia, researchers have recently argued that it is a critical component of CKD progression and therefore a key therapeutic target.[4,10]

All of the discussed factors, hyperfiltration, proteinuria, tubulointerstitial inflammation, oxidative damage and induction of the RAAS contribute to disruption of tubuloglomerular continuity, chronic hypoxia, further inflammation, and fibrosis, which is integral to the progressive, irreversible nature of CKD. Many of these factors likely have a negative effect on telomere length, either directly, or indirectly through cell damage and resulting repair and replication. An environment of continued damage in an already aging kidney likely also exacerbates cellular senescence. Understanding the pathophysiology of CKD allows exploration of potential therapies such as mesenchymal stem cell (MSC) therapy; aimed specifically at amelioration of the inflammatory component of the disease.

### *1.1.3 Markers Associated with Renal Inflammation, Fibrosis and Vascular Health*

Cytokines and chemokines are released by damaged glomerular, mesangial and endothelial cells, activated tubular cells, and infiltrating inflammatory cells and are thought to be critically involved in the progression of renal inflammation. Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine that is thought to play a critical role in fibrosis. TGF-β1 is released from parenchymal cells and inflammatory cells and can result in production of extracellular matrix proteins and differentiation of tubular epithelial cells into myofibroblasts.[11] Urinary TGF-β1 levels are correlated with renal parenchymal TGF-β1 and the degree of interstitial fibrosis in humans.[12,13] In one previous study in cats,

urinary TGF-β1 levels were found to be elevated in cats with CKD.[14] Interleukin-8 (IL-8) is a chemokine released by white blood cells and endothelial cells in response to inflammation that recruits neutrophils to sites of inflammation. Elevated IL-8 concentrations have been correlated with pyelonephritis in children as well as the subsequent degree of renal fibrosis.[15] Monocyte chemoattractant protein-1 (MCP-1) is another chemokine that is expressed at sites of injury or inflammation that functions to recruit inflammatory monocytes.[16] Elevated concentrations of MCP-1 have been associated with degree of inflammatory infiltrate, proteinuria, and severity of disease in humans.[12,16] VEGF, a potent pro-angiogenic factor important in heath and repair of renal vasculature, has been shown to be down-regulated in end stage renal disease in humans and is thought to be a useful prognostic biomarker for loss of renal function.[10] In previous studies in humans urinary VEGF excretion was reduced in more severe kidney disease.[10,17]

### *1.1.4 Urinary Cytokines for Assessment of CKD*

Recently, several studies have provided evidence that urinary cytokines may be useful as a non-invasive assessment of renal disease and may provide an additional method by which progression of disease and response to therapy could be monitored.[15,18-20] We recently performed a study comparing urinary cytokine levels in normal cats and cats with CKD as background information that would allow us to better understand urinary cytokine levels measured in cats enrolled in the mesenchymal stem cell trials discussed in Chapter 5. When urine cytokine concentrations in healthy and CKD cats were compared, we found significantly higher concentrations of IL-8, MCP-1, and TGF-β1 in urine of CKD cats (**Figure 1.1-1.3**), along with significantly lower VEGF concentrations (**Figure 1.4**). A significant positive correlation between serum creatinine and urinary IL-8 and TGF-β1 concentrations was found, as well as a negative correlation between serum creatinine and urinary VEGF concentrations. Urinary cytokine measurement may be a potentially useful means of assessing intra-renal inflammation, fibrosis and vascular health in

cats with CKD. With this premise, urinary cytokines were used to monitor MSC clinical trial cats in an effort to determine if MSC were having an effect on intrarenal inflammation, fibrosis and vasculature.

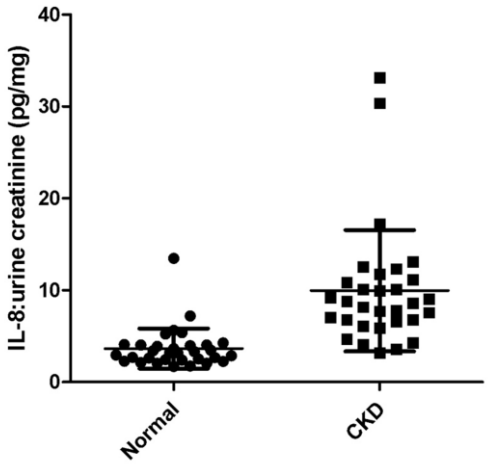



**Figure 1.1:** Urinary IL-8:urine creatinine ratio of healthy (n = 32) and CKD (n = 32) cats as measured by ELISA. Urine from CKD cats had a significantly higher IL-8:UCrR than urine from healthy cats (Mann-Whitney: $p<0.0001$).

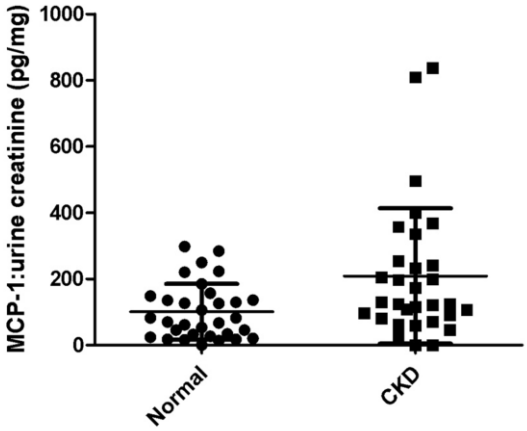



**Figure 1.2:** Urinary MCP-1:urine creatinine ratio of healthy (n =32) and CKD (n = 32) cats as measured by ELISA. Urine from CKD cats had a significantly higher MCP-1:UCrR than urine from healthy cats (Mann-Whitney: $p<0.007$).

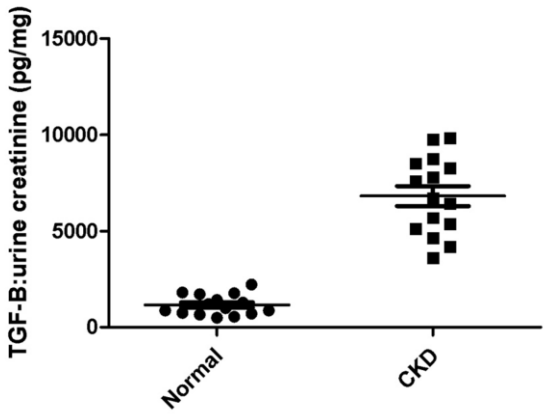



**Figure 1.3:** Urinary TGF-β1: urine creatinine ratio of healthy (n = 15) and CKD cats (n = 15) as measured by ELISA. Urine from CKD cats had a significantly higher TGF-β1:UCrR than urine from apparently healthy cats (Mann-Whitney: p=.0002).

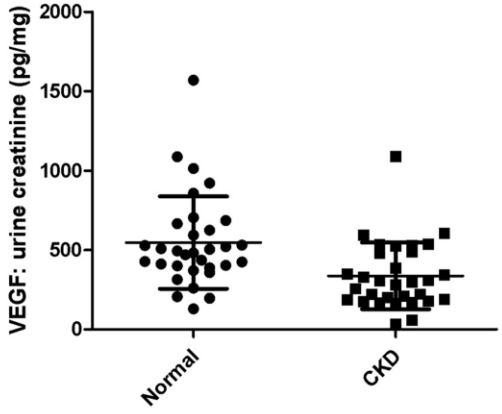



**Figure 1.4:** Urinary VEGF: urine creatinine ratio of healthy (n = 32) and CKD cats (n =32) as measured by ELISA. Urine from CKD cats had a significantly lower VEGF:UCrR than urine from apparently healthy cats (Mann-Whitney: p=.0018).

## 1.2 Renal Senescence and Telomere Biology

### *1.2.1 Kidneys and Age*

The phenotype of renal aging is described not only as the loss of function and renal mass, but as the loss of an appropriate response toward injury.[21] In humans there is progressive loss of renal parenchyma and function with age, and the kidney is one of the organs that ages the fastest.[22] In the aging human population kidney injury is more common, clinically more severe, and kidney function is less likely to recover.[23] In addition, age is the most important predictor of renal transplant longterm success; older donor kidneys are more likely to develop allograft nephropathy.[24-26] Because of these factors, the study of renal aging has garnered much interest. The exact molecular mechanisms of renal aging have not been elucidated, but theories of aging include oxidative damage, genomic instability (including telomere loss), genetic programming (such as senescence) and cell death.[27]

Senescence is a fundamental cellular program similar to apoptosis and results in an organs inability to repair and replicate, particularly in response to stresses. Senescence is associated with telomere shortening, in which case it is usually referred to as replicative senescence, but this is not the only reason it happens. Other factors may include DNA damage, oxidative stress and response to other stresses. Senescence is mediated by p16 and p53 signaling pathways.[28]

Several studies have examined aging kidneys and their association with senescence. $P16^{INKA4a}$ is an irreversible cell cycle inhibitor which is considered to be one of the best markers for senescence.[27] When human biopsy specimens over a large age range were examined for genes associated with senescence, P16 expression, particularly in the renal cortex, was found to be most consistently correlated with age as well as histologic changes, and was inversely correlated with cell replication.[27] Renal senescence does appear to involve more than telomere shortening as was demonstrated in one study where telomere length, as assessed by telomere restriction fragment (TRF) analysis, was not found to shorten in the kidney with age even though increased markers of senescence, $P16^{INKa4a}$ and B gal, were shown.[29] In another study, diseased kidneys were confirmed to have evidence of increased senescence. When human biopsy specimens were examined, diseased kidneys (affected by glomerulopathies)

expressed more $P16^{INK4a}$ than expected for their age in all compartments including the tubules, the glomerulus and the interstitium.[26]

Senescence in renal age and disease has a number of critical effects on the function of the kidney. This results in a diminished capacity of the aged kidney to cope with or withstand normal and abnormal stresses. The capacity for renal epithelial cell proliferation declines with aging, both baseline cell proliferation as well as capacity for bursts of proliferation after injury.[30] Susceptibility to apoptosis also increases with age in both baseline and crisis conditions. Additionally it has been demonstrated that function of stem cells and progenitor cells decrease with age.[30] The kidney's ability to provide vital growth factors is also diminished with age and disease. Senescent cells secrete altered levels of TGF-B, EGF, IGF-1 and VEGF. VEGF is critical for vascular health and repair and reduced expression in aged and diseased kidneys likely results in the progressive rarefaction of peritubular capillaries and hypoxia that has been observed.[10,30] Reaching a better understanding of the mechanisms of renal aging and disease is a critical first step on the pathway to developing mechanism specific therapies for amelioration of age and disease related effects.

### *1.2.2 Telomeres and Aging*

Telomeres are protective structures on the end of chromosomes. The name is derived from two greek nouns telos "end" and meros "part". They cap the chromosome and prevent the end from being recognized as a DNA strand break. DNA strand breaks lead to cell cycle arrest and repair, or apoptosis if the damage is severe. Telomeres prevent such events and are critical for chromosome stability.[31] Unfortunately telomeres shorten with each replication cycle due to "end replication problem"; DNA polymerase cannot fully replicate to the very end of the DNA and with each cycle some is lost. Additional factors can also lead to telomere shortening (additions, deletions, oxidative damage).[31,32] Telomerase is an enzyme that can compensate for this and create new telomeres *de novo*, but it is generally not active in somatic cells after embryogenesis. Telomerase can be reactivated in cancer.[31]

When telomeres reach a critically short state, they are no longer able to perform the capping function and are themselves seen as a DNA strand break. At this point, p53 and p21 may be activated, cell cycle arrest occurs, and ultimately cell death.[33] Most tissues in humans have been documented to experience telomere shortening as a matter of age; including peripheral blood mononuclear cells (PBMC), kidney, endothelial cells, hepatocytes, intestinal epithelium, lung, and muscle.[31] Telomeres have also been shown to be shortened in several disease states.[32] Some genetic mutations have been shown to lead to critical telomere dysfunction.

Telomeres are acutely sensitive to oxidative stress, in part due to their high content of guanines but also because reactive oxygen species (ROS) produce single strand breaks and telomeric DNA is thought to be deficient in the repair of single strand breaks.[32] Additionally, repair of oxidative lesions appears to be less efficient than in the rest of the genome.[32] Several studies provide evidence that reduction of oxidative stress via antioxidant treatment may reduce telomere attrition and delay cellular senescence.[34,35] There appears to be an association between inflammation, oxidative stress and telomere shortening and thus investigation of telomere length is pertinent in any inflammatory disease condition, including chronic kidney disease.

### *1.2.3 Telomeres and the Kidney*

Several studies have assessed the association between renal age, renal disease, and telomere length in humans and rats. One study explored the relationship between age and telomere length in human surgical biopsy samples.[36] TRF and slot blot analysis were used to assess telomere length; the results of these methods were found to be comparable. Telomere loss in the cortex occurred with age at a rate of 0.25% per year. Telomere loss in the medulla was not significant. TRF length was greater in the renal cortex than the medulla, with the difference being greater in young kidneys and lessening with age due to telomere loss in the cortex. It was concluded that telomeres shorten in an age-dependent manner, particularly in the renal cortex.[36] In another study age-dependent telomere attrition rate was measured in the outer renal cortex and inner renal medulla of Caucasians and African Americans.[37] Telomere length was measured by

TRF analysis. TRF length attrition rate was slower in the inner medulla than in the outer renal cortex in both groups. In the outer renal cortex, TRF length attrition rate was significantly slower in African Americans than Caucasians. The proximal tubule is the most abundant nephron structure in the renal cortex and it was concluded these findings may represent a difference in proliferative growth between racial groups.[37]

The importance of functional telomeres to kidney repair has also been explored. In an intriguing study, renal ischemia-reperfusion injury led to greater impairment of renal function and increased acute and chronic histopathologic damage in fourth generation telomerase-deficient mice compared with wild-type and first generation mice.[21] Critically short telomeres, increased expression of cell cycle inhibitor p21 and more apoptotic renal cells were associated with the increased damage in fourth generation mice. These mice also had reduced proliferative capacity, highlighting the detrimental effect telomere shortening has on replication and repair.[21]

It has also been shown that other factors, such as proteinuria and hypertension, which are common in chronic kidney disease, have a detrimental effect on telomere length. In rats with genetic spontaneous hypertension, telomere length as measured by TRF was found to be shorter in all ages in comparison to normal type rats. It was concluded that hypertension resulted in increased cell turnover and therefore accelerated cell aging.[38] In a study assessing differences between longevity in male vs. female proteinuric rats, it was found that increased proteinuria in male rats was associated with shortened renal telomeres, increased expression of senescence markers p21 and p53, and reduced oxidative damage protective mechanisms.[39] More specifically, telomere shortening occurred with age in rats, but was more significant in male rats and was also accelerated in the renal cortex of both sexes. It was concluded that decreased antioxidant capacity and increased proteinuria in male rats may induce accelerated telomere shortening leading to senescence, renal failure and decreased longevity.[39]

Although it has been demonstrated that many factors negatively affect telomere length, there is some indication that therapeutic intervention can provide a protective effect. In an *in vitro* study the angiotensin II receptor blocker losartan was demonstrated to have a protective effect on cultured

glomerular mesangial cells exposed to angiotensin II.[33] Cells which were exposed to angiotensin II plus losartan had longer telomere lengths, and decreased senescence as measured by B-galactosidase staining and expression of p21 and p53, than cells exposed to angiotensin II alone.[33] Although yet to be demonstrated *in vivo,* this type of therapeutic manipulation of telomere length provides encouragement that other potential therapies remain to be explored.

### *1.2.4 Telomeres and Cats*

Cats have 38 chromosomes and it was demonstrated with the use of fluorescence *in situ* hybridization (FISH) that telomeres were present on the terminal portion of the chromosome of cultured feline peripheral blood mononuclear cells (PBMC).[40] It was observed that although telomeres were present on all chromosomes, the signal varied in intensity between homologues. Telomere length in relation to age in cats was investigated in another study.[41] TRF analysis was used to measure telomere length in the PBMC of 30 DSH as well as the tissues of 2 young DSH. Telomerase activity was also assessed. A significant decrease in telomere length in PBMC was found with age in cats. This has also been found to be true in dogs and humans.[41] Telomerase activity was not found in a wide range of normal tissues. The authors conclude that telomere length is implicated in the aging process in cats.[41] Telomere length and the pattern of expression of telomerase in cat tissues is similar to that in humans (in contrast to mice) and cats may serve as a translational model for studies of aging and telomere biology. Telomere length has not been previously assessed in the feline kidney or in feline chronic kidney disease.

### *1.2.5 Diagnostic Methods*

*Telomere Length*

Several methods have been developed for the measurement of telomere length in biological samples. Each method differs slightly in its potential uses and limitations. Perhaps the most commonly used techniques are TRF analysis and different variations of FISH such as quantitative FISH (Q-FISH) and flow cytometry combined with FISH (flow-FISH).[42,43] TRF by southern blot is a relatively simple assay

and is probably the most widely used in telomere research. In this method average lengths of terminal restriction fragments are measured by cutting DNA into fragments by restriction enzymes, separating them by electrophoresis and then hybridizing the DNA fragments with labeled probes specific for telomere sequences. However disadvantages include requirement for a relatively large amount of DNA from unfixed samples, inability to distinguish between cell populations in the target tissue, failure to measure actual telomere length, and some subjectivity to analysis of the autoradiographic smears produced by the method.[42,43] FISH technology, using telomere-specific oligonucleotide probes avoid many of the problems encountered in TRF. In this method telomere length can be measured in single cells by hybridization with a fluorescent probe which allows telomeres to be visualized and quantified by measuring light intensity. In Q-FISH quantitative measurement of telomere length is even possible using metaphase spreads, however this limits analysis to replicating cells. Smaller amounts of sample are needed for FISH analysis but analysis of fixed tissues or specific cell phenotypes is still not possible.[43]

As an alternative to previously described methods, a relatively new FISH technique involves a combination of FISH and immunostaining (TELI-FISH) so that telomere length in specific cell populations within a tissue sample can be measured.[43] This is particularly advantageous in tumor biology as it allows normal cell populations to be distinguished from neoplastic cell populations within the same tissue sample. An additional benefit of this method is the ability to utilize paraffin-embedded tissues. We adapted this particular technique to facilitate measurement of telomere length in specific cell populations in the feline kidney.

*Renal Immunohistochemistry*

In order to perform TELI-FISH on samples in this study and identify specific cellular populations in the renal cortex, antibodies for immunohistochemistry had to be identified. Previously Brandt *et al* investigated localization of renal membrane proteins in the cat and dog and pinpointed key antibodies for identification of different regions of the nephron.[44] Aquaporin 1 was discovered to be specific to the

proximal tubule and glomerulus in the cat. Immunohistochemistry of the renal cortex demonstrates prominent staining of proximal tubular structures.[44]

The second immunohistochemistry antibody that was selected for TELI-FISH is cytokeratin. Cytokeratin AE1/AE3 is considered to be a pancytokeratin antibody which identifies most epithelial cells. However this product is a mixture of anti-cytokeratin monoclonal antibodies AE1 and AE3 which stain almost all cytokeratins (1-19) except for cytokeratin 18. Proximal tubular epithelial cells are cytokeratin 18 positive and cytokeratin 8 negative while distal tubular epithelial cells are cytokeratin 8 positive.[45] The result is that cytokeratin will only identify distal tubular segments and not proximal tubular segments.[45]

## 1.3 Nutritional Management of Cats with Chronic Kidney Disease

### *1.3.1 Introduction*

Clinical signs of feline CKD include polyuria, polydipsia, decreased appetite, weight loss and vomiting. Generally at the time that routine laboratory tests reveal the presence of kidney dysfunction, approximately 75% of renal function is already lost. Currently no treatment short of renal transplantation has been shown to reverse or halt declining renal function for any significant period of time. Unfortunately renal transplant is not widely available and is too expensive for many clients. Additionally not all CKD cats are acceptable candidates for transplantation. Medical management therefore is the mainstay of treatment and can help patients cope with metabolic complications of the disease and improve quality of life.[2,46] Metabolic complications and imbalances such as azotemia, hypertension, proteinuria, hypokalemia, hyperphosphatemia, anemia and dehydration progressively worsen with disease stage and may affect appetite. Management of these complications is a core part of medical therapy for these patients.[2] Nutrition is important for long term prognosis and efforts to directly target nausea and appetite, in addition to other medical therapies, will undoubtedly benefit patients.

### *1.3.2 The Importance of Nutrition*

Management of long term hyporexia is important. Poor body condition is associated with decreased prognosis in several species and has a negative effect on immune function, wound healing and strength.[47-49] In a recent study in dogs with CKD, poor body condition was associated with shortened median survival time.[48] Most importantly poor appetite also has a negative effect on owner perception of quality of life and can result in a great deal of stress. Cats have a higher requirement for protein and amino acids than other species. When nutrition is inadequate, energy is derived from mobilization of amino acids from muscles stores as opposed to fat.[47] Elderly cats are also unique in comparison to other species as they have stable to increased level of metabolism, as opposed to decreased metabolism.[50] A reduced ability to digest protein and fat has been documented in elderly cats.[51] These combined factors make the utilization of high quality, easily digestible food product critical for these patients. Cats are also particularly sensitive to changes in environmental factors, such as the timing and location of feeding, as well as the food type; smell and "mouth feel" may also play into their willingness to eat.[52]

A meal can be thought of as having three parts; initiation, maintenance and termination. Different stimuli are responsible for each phase. Initiation of a meal depends heavily upon environmental stimuli such as olfactory, visual, tactile and auditory cues.[52] Termination of a meal occurs as a result of negative feedback mechanisms such as gastric distention or presence of nutrients in the intestine. Suppression of appetite may occur as a result of psychological, environmental or physical factors such as decreased olfaction, nausea, pain, fever, ileus, or vomiting that interfere with this process.[47] Circulating inflammatory mediators such as TNF-a, IL-1, and IL-6 may play a role in suppression of appetite.[47]

Care should be taken to select the appropriate patients for appetite enhancement as learned food aversion is thought by most to be prevalent in cats.[52] Learned food aversion occurs when the patient associates nausea, pain or other physical manifestations of disease with the act of eating or with the sight or scent of food. Even after the underlying illness is resolved, this aversion may remain. Therefore it is critical that cats that are overtly nauseous – drooling, gagging, turning away from food - particularly in

hospital or in acute illness are not forced to eat lest food aversion be created.[52] If cats are too nauseous or critical to even consider oral feeding, or have not responded to appetite encouragement after 3-5 days, placement of an enteral feeding tube should be considered. Nasoesphageal, esophageal or gastrotomy tube can be chosen depending on the type and duration of feeding desired.[47] Parenteral feeding should be considered in cats that cannot tolerate enteral feeding. Additionally many clinicians feel that prescription diets (i.e. renal) should not be fed in hospital during a crisis lest an aversion be created to the diet desired for long term management. The best candidates for pharmacological enhancement of appetite are cats leaving the hospital with their acute crisis resolved, and cats with chronic disease in the home environment; such as cats with chronic kidney disease.

### *1.3.3 Nutritional Therapies*

*Diet*

Several recent studies have documented the therapeutic value of specially formulated diets in the management of CKD.[53-55] In Ellliot et al 2000, cats who were prospectively placed on a renal diet experienced a reduction in serum phosphorus and urea as well as prevention of elevated PTH levels. Furthermore cats in this group had a median survival time of 633 vs. 264 days for cats on a maintenance diet.[53] In a retrospective study comparing survival time between cats that were fed commercial maintenance diets vs. renal diets, cats that ate renal diets had a median survival time of 16 months vs. a median survival time of 7 months.[54] In a third randomized masked clinical trial, cats were assigned to a renal diet or adult maintenance diet. In this study serum urea nitrogen levels were lower in cats fed renal diet and bicarbonate concentrations were higher. Additionally cats fed the renal diet had significantly fewer uremic crises (0% vs 26%) than cats fed the maintenance diet and fewer cats fed the renal diet died of renal related illness.[55] Commercially formulated renal diets typically contain restricted amounts of high quality protein, adequate non-protein calories, and are restricted in phosphorus. Unfortunately cats with CKD often suffer from poor appetite. The failure of the patient to eat the diet negates the benefit of

dietary management, and therefore a key therapeutic target for these patients is the maintenance of appetite and food intake.

*Anti-Nausea Therapy*

Cats with CKD likely suffer from nausea, vomiting and inappetence as a result of uremia, a buildup of toxins in blood, that affects the chemoreceptor trigger zone in brain. In addition, uremia may have effects on the intestinal tract that lead to further unwillingness to eat. Several anti-nausea therapies have become recently available. These include maropitant, ondansetron, dolasetron and mirtazapine. These drugs work at the nausea center in the brain as well as in the gut and some can be given as an injection. Ondansetron has been documented to be helpful in human patients suffering from uremia but has not been evaluated in a study in cats.[56] Mirtazapine demonstrates anti-nausea properties in addition to its appetite stimulating properties as it acts at the 5-hydroxytryptamine $(5\text{-HT})_3$ receptor similarly to ondansetron. It has been shown to reduce nausea and vomiting associated with psychological disorders, cancer chemotherapy, anesthesia, and intra-thecal injections in humans.[57-60] Its anti-nausea properties have not previously been evaluated in veterinary species. Limiting gastric acidity with the use of $H_2$ blockers such as famotidine anecdotally appears to palliate inappetence in some patients, however both the degree of hyperacidity present in CKD and the efficacy of these medications remain unproven both in human and veterinary patients.

*Appetite Stimulant Therapy*

Prior to the use of mirtazapine in veterinary medicine, there were few other pharmacological options for appetite stimulation. Cyproheptadine has been used for some time as an appetite stimulant and has anecdotal efficacy, however this efficacy has never been scientifically evaluated for veterinary patients. Twice daily administration is necessary in many cases and this can prove a challenge for owners, particularly longterm. Diazepam was previously used, both intravenously and orally, but oral use has been

associated with fatal hepatotoxicity and is not recommended.[61] No previously literature is available regarding efficacy of these appetite stimulants.

### *1.3.4 Mirtazapine as a Novel Therapy*

Mirtazapine was originally introduced to human medicine as an antidepressant; however it has recently attracted interest in veterinary medicine due to several desirable effects, namely its significant anti-nausea, anti-emetic, and appetite stimulating properties. Mirtazapine is a pre-synaptic $\alpha$2-adrenergic receptor antagonist that increases noradrenergic and serotonergic neurotransmission by blocking pre-synaptic inhibitory receptors, resulting in increased norepinephrine release into the synaptic cleft and therefore increased post-synaptic availability.[62] The serotonergic effects occur through 5-$HT_1$ receptor-like activity as well as enhancement of serotonergic transmission by norepinephrine. In contrast to these effects, mirtazapine is also a potent post-synaptic 5-$HT_3$ and 5-$HT_2$ antagonist. It is thought that the receptor-specific anti-serotonergic effects help ameliorate side-effects of the drug.[62] Although the original mechanism of action studies were performed in rats, and no information is available for cats, mirtazapine likely exerts its purported effect through analogous mechanisms in the species of interest. These effects appear to be a result of antagonism of the 5-$HT_3$ receptor, which is an important receptor in the physiology of emesis. Noradrenergic effects appear to be responsible for the antidepressant properties.[63]

Mirtazapine has been previously evaluated as an appetite stimulant in human medicine. In a Phase II trial for cancer patients suffering from anorexia and cachexia, mirtazapine was found to improve quality of life and result in weight gain in at least 25% of patients. The study was plagued by a high dropout rate due to the severity of disease.[64] In veterinary medicine, an initial uncontrolled clinical trial in cats and dogs[65] and numerous anecdotal reports have encouraged clinical use of mirtazapine. In the uncontrolled clinical trial 24 dogs and 17 cats with varying medical conditions were treated with doses extrapolated from human medicine. Mirtazapine therapy led to an excellent response in 12 cases, a better response than standard therapy in 16 cases and an equivocal response in 13 cases. It was thought that the best responses were seen in animals suffering from kidney disease or undergoing chemotherapy. A dose

of 1.88 or 3.75 mg (1/8 or 1/4 of a 15 mg tablet) every three days was recommended for cats,[65] however no pharmacological studies were performed to support these extrapolations. Side effects did not include somnolence as seen in people, but rather increased activity. Higher doses in cats were associated with muscle tremors, but otherwise the medication appeared well tolerated. In response to the publication of this trial and other anecdotal reports, mirtazapine began to be used commonly in our practice. However, anecdotal observations by the authors noted that in most cases the effect of the drug appears to have dissipated by the second day after administration, implying a shorter dosing interval might be more appropriate.

Human pharmacokinetic data have demonstrated that a number of factors affect the metabolism of mirtazapine. Differences in metabolism were found between sexes and ages, and for patients suffering from hepatic or renal impairment.[63] Effect of renal impairment on mirtazapine pharmacokinetics was assessed in three groups of human patients with mild, moderate and severe renal disease. It was discovered that the area under the curve (AUC) was increased in moderately and severely affected patients, but not mildly affected patients in comparison to controls. The elimination half-life was not affected by the degree of renal impairment, but was longer in the severely affected group in comparison to healthy controls. The clearance of mirtazapine was decreased 33% in moderately affected individuals and 50% in severely affected individuals.

## 1.4 Novel Therapies For Chronic Kidney Disease: The Potential of Mesenchymal Stem Cells

Regenerative medicine refers to the process of using living functional tissues to repair or replace organs that are functionally damaged. Stem cell therapy in particular is an innovative new field of scientific investigation and clinical application that holds promise for a variety of diseases in veterinary medicine as well as human medicine. Recent years have brought increased interest in the potential for adult stem cells to help in the treatment of many diseases through both their regenerative properties as well as their apparent ability to alter the environment in injured and diseased tissues. In particular, adult

stem cells called mesenchymal stem cells can migrate to affected areas and may be able to support the growth of other stem cells as well as modulate the response of the immune system.

A stem cell is a generic term referring to any unspecialized cell that is capable of long-term self-renewal through cell division but that can be induced to differentiate into a specialized, functional cell. Stem cells are generally divided into two groups, embryonic stem cells and adult stem cells. Adult stem cells can be obtained from many differentiated tissues including but not limited to bone marrow, bone, fat, and muscle. Obtaining adult stem cells also does not raise ethical concerns, and most commonly stem cells are obtained from bone marrow or adipose sources. For most studies, the adult stem cell in question is actually a mesenchymal stem cell or mesenchymal stromal cell (MSC). MSC are multipotent but not pluripotent, which means they can differentiate into some, or "multiple," but not all tissue types.[66]

### *1.4.1 Characterization*

MSC are plastic adherent and assume a fibroblast-like morphology during culture. They proliferate easily in culture and can be cryopreserved without loss of phenotype or differentiation potential.[67] Additionally cell surface marker characterization via flow cytometry differentiates them from hematopoetic cells, though no truly unique MSC molecule has been identified.[68] For the most part, MSC have been reported to be CD44 postive, CD 90 positive, CD 73 postive, CD 105 positive, CD 45 negative, and HLA-DR negative.[68-71] In part, the lack of definitive markers probably reflects the diverse lineage of MSC and the fact that each MSC population reflects to some degree the characteristics of tissues from which they were derived. Most importantly stem cells from both adipose and bone marrow sources possess the ability to differentiate into cell types of multiple lineages including adipocytes, chondrocytes, and osteocytes.[66,68]

### *1.4.2 Immunologic Properties*

Mesenchymal stem cells clearly modulate immune responses, as demonstrated by both *in vitro* and *in vivo* studies. For example, MSC are poor antigen presenting cells and do not express MHC class II or co-

stimulatory molecules and express only low levels of MHC class I molecules.[66] Thus, MSC are very non-immunogenic and can be transferred to fully allogeneic recipients and still mediate their immunologic effects.[72] Among their other immunological properties, MSC inhibit lymphocyte proliferation and cytokine production, suppress dendritic cell (DC) function and alter DC cytokine production, and decrease IFN-g production by NK cells.[66] *In vitro* studies have demonstrated that MSC can produce growth factors, cytokines, and anti-inflammatory mediators, all of which could help maintain or improve renal function and suppress intra-renal inflammation.[73-75] The ability of MSC to suppress inflammation appears to be mediated both by secreted factors and by direct contact with inflammatory cells.[74,75] Thus, MSC have the potential to suppress intra-renal inflammation. These properties of MSC could potentially be harnessed therapeutically.

### *1.4.3 MSC and Chronic Kidney Disease*

There are numerous studies of MSC therapy in rodent models of renal failure, though most studies have focused on models of short-term protection from acute kidney injury (AKI).[73,76-78] The majority of these studies provide evidence that systemic administration of bone marrow-derived or adipose tissue-derived MSC can help preserve renal function in the face of acute insults, such as ischemic injury, toxic insult and obstruction, and can also help reduce tubular injury and fibrosis.[73,76-78] Several studies have also demonstrated incorporation of small numbers of MSC into the renal parenchyma.[77,79,80] It has been proposed that some of these MSC may actually differentiate into functional renal tubular epithelial cells, though this theory remains controversial. Other investigators propose that paracrine effects from the injected MSC are more important than the effects of direct cellular incorporation into the kidney.[73,81] Thus, the available data indicate that systemically administered MSC can help improve or stabilize renal function in acute renal disease by a variety of mechanisms.

Fewer studies have investigated the effects of MSC therapy in chronic renal failure models in rodents.[69,70,82-86] In the CKD rodent models that have been investigated, administration of MSC has been beneficial, especially with respect to reducing intra-renal inflammation and suppressing fibrosis and

glomerulosclerosis.[69,70,82,86] The results of several studies, which all offer slightly different viewpoints, are summarized here to provide an overview of the current state of knowledge in regards to rodent models.

In a murine model of genetic CKD, collagen 4A3 deficiency, weekly injection of bone marrow derived MSC into the tail vein was shown to decrease interstitial fibrosis and preserve peritubular capillaries, but renal failure and death were not delayed.[86] Injected MSC were localized to the peritubular capillaries and were not observed to differentiate in to renal cells. One mechanism of peritubular capillary preservation was thought to be MSC secretion of VEGF; basal secretion of VEGF by MSC was shown, as well as a two fold increase in VEGF expression in MSC treated kidneys.[86]

One study examined the effects of intraparenchymal injection of human bone marrow derived MSC and human fetal kidney cells into rats with experimentally induced CKD and AKI.[84] This study provided a particularly useful model for induction of CKD as the researchers waited 6 weeks after renal injury to start interventions, allowing for more chronicity of the disease, compared to several other studies. Fetal kidney stem cells were shown to have no effect in comparison to MSC. MSC were injected once directly into the kidney in CKD rats and resulted in restoration of normal kidney function for approximately 2 months before relapse. MSC injected in AKI rats resulted in improved renal function and prevented death from uremia. No histopathology was evaluated in this study.[84]

In another 5/6 nephrectomy rat model, one dose of bone marrow derived MSC from male rats was given via the tail vein to female rats only 1 day after surgical manipulation.[85] No significant differences in renal function or histopathology were seen between groups, but the MSC treated group experienced increased weight and decreased proteinuria. Increased expression of VEGF in treated kidneys was also demonstrated one month after injection; it then subsequently decreased. Y chromosome FISH was also performed to evaluate presence of male MSC in female kidney tissue at 21 days. These authors argued for engraftment of MSC based on the presence of Y chromosome positive cells.[85]

Subcapsular injection as a route of MSC administration was explored in another 5/6 nephrectomy rat model.[70] After a single injection of bone marrow derived MSC into the subcapsular area in rats, significant reductions in renal values, blood pressure and proteinuria were seen, as well as a reduction in

glomerulosclerosis. Migration of cells into the renal parenchyma from the area of injection was also observed.[70]

In a pivotal and well executed study, Semedo et al. demonstrated that administration of MSC attenuates fibrosis in a rat 5/6 nephrectomy model.[69] This study compared the effect of a single IV MSC injection to a series of three IV MSC injections given every other week, with treatments starting two weeks after surgical manipulation. Fibrosis was significantly decreased in animals that received multiple injections, but was also slightly improved in animals that got a single injection. Renal function, proteinuria and hematocrit were significantly improved in the multiple injection group, but not in the single injection group. Inulin clearance and blood pressure were not significantly improved with any treatment. MSC were also localized to the kidney after IV administration, and were found in interstitial areas and near vessels. However the researchers still argued that MSC effects are paracrine in nature and a variety of cytokines were also assessed in this study. Pro-fibrotic molecules and cytokines and pro-inflammatory cytokines were found to be decreased in the multiple injection group, specifically TGF-B, MCP-1 and IL-6. Our IV allogeneic clinical trial was designed based on the methodology of this particular study.[69]

Subsequently an additional study also demonstrated the advantage of repeated IV administration vs single IV administration of MSC in a rat 5/6 nephrectomy model.[82] Weekly administration of bone marrow derived MSC was compared to single injection of MSC or mesangial cells. Repeated MSC administration lead to improvement in renal function, blood pressure and proteinuria, whereas improvement was not seen in other treatment groups. Some decrease in renal fibrosis and inflammatory infiltrate was seen with single MSC administration, but weekly MSC administration resulted in a much more significant improvement. Although MSC were localized to the kidney one week after IV injection, they were not found 5 weeks after IV injection. Cytokine effects were also assessed and only weekly MSC administration lead to a decrease in renal cortical expression of IL-6 and an increase in IL-10.[82]

Although literature for the efficacy of a single MSC injection has reported variable results, another recent study did find benefit in a single IV injection of MSC.[83] In this study bone marrow derived

MSC were injected via the tail vein at the time of nephrectomy (making this a less optimal CKD model). Renal function and histopathologic characteristics of damage were improved as a result of MSC injection. Pro-fibrotic molecules and cytokines were reduced and VEGF expression was increased in MSC injected rats.[83]

In summary, MSC therapy for CKD in rodent models of genetic and experimentally induced disease has indicated significant renoprotective effects, including reduction of intrarenal inflammatory infiltrates, decreased fibrosis and decreased glomerulosclerosis. Several routes of administration – intrapararenchymal, subcapsular, IV - have been used and all seem to be effective. MSC effects appear to come both from anti-inflammatory properties as well as protection of vascular integrity as mediated by VEGF. However, results of rodent models should be evaluated with care as administration of MSC immediately after surgical nephrectomy is not representative of long standing naturally occurring disease. A naturally occurring model of CKD, such as that which occurs in cats, has not been scientifically evaluated at this time.

## 1.5 References


**1.** Boyd LM, Langston C, Thompson K, Zivin K, Imanishi M. Survival in cats with naturally occurring chronic kidney disease (2000-2002). *J Vet Intern Med.* Sep-Oct 2008;22(5):1111-1117.

**2.** Polzin DJ. Chronic kidney disease in small animals. *Vet Clin North Am Small Anim Pract.* Jan 2011;41(1):15-30.

**3.** Bohle A, Mackensen-Haen S, von Gise H, et al. The consequences of tubulo-interstitial changes for renal function in glomerulopathies. A morphometric and cytological analysis. *Pathol Res Pract.* Feb 1990;186(1):135-144.

**4.** Nangaku M. Chronic hypoxia and tubulointerstitial injury: a final common pathway to end-stage renal failure. *J Am Soc Nephrol.* Jan 2006;17(1):17-25.

**5.** Harris RC, Neilson EG. Toward a unified theory of renal progression. *Annu Rev Med.* 2006;57:365-380.

**6.** Siragy HM, Carey RM. Role of the intrarenal renin-angiotensin-aldosterone system in chronic kidney disease. *Am J Nephrol.* 2010;31(6):541-550.

**7.** Rodriguez-Iturbe B, Garcia Garcia G. The role of tubulointerstitial inflammation in the progression of chronic renal failure. *Nephron Clin Pract.* 2010;116(2):c81-88.

**8.** Sanchez-Lozada LG, Tapia E, Johnson RJ, Rodriguez-Iturbe B, Herrera-Acosta J. Glomerular hemodynamic changes associated with arteriolar lesions and tubulointerstitial inflammation. *Kidney Int Suppl.* Oct 2003(86):S9-14.

**9.** Keegan RF, Webb CB. Oxidative stress and neutrophil function in cats with chronic renal failure. *J Vet Intern Med.* May-Jun 2010;24(3):514-519.

**10.** Mayer G. Capillary rarefaction, hypoxia, VEGF and angiogenesis in chronic renal disease. *Nephrol Dial Transplant.* Apr 2011;26(4):1132-1137.

**11.** Branton MH, Kopp JB. TGF-beta and fibrosis. *Microbes Infect.* Dec 1999;1(15):1349-1365.

**12.** Bobkova IN, Chebotareva NV, Kozlovskaia LV, Varshavskii VA, Golitsyna EP. [Urine excretion of a monocytic chemotaxic protein-1 and a transforming growth factor beta1 as an indicator of chronic glomerulonephritis progression]. *Ter Arkh.* 2006;78(5):9-14.

**13.** Murakami K, Takemura T, Hino S, Yoshioka K. Urinary transforming growth factor-beta in patients with glomerular diseases. *Pediatr Nephrol.* Jun 1997;11(3):334-336.

**14.** Arata S, Ohmi A, Mizukoshi F, et al. Urinary transforming growth factor-beta1 in feline chronic renal failure. *J Vet Med Sci.* Dec 2005;67(12):1253-1255.

**15.** Sheu JN, Chen SM, Meng MH, Lue KH. The role of serum and urine interleukin-8 on acute pyelonephritis and subsequent renal scarring in children. *Pediatr Infect Dis J.* Oct 2009;28(10):885-890.

**16.** Eardley KS, Zehnder D, Quinkler M, et al. The relationship between albuminuria, MCP-1/CCL2, and interstitial macrophages in chronic kidney disease. *Kidney Int.* Apr 2006;69(7):1189-1197.

**17.** Bobkova IN, Kozlovskaia LV, Rameeva AS, Varshavskii VA, Golitsyna EP. [Clinical implication of urine test for markers of endothelial dysfunction and angiogenesis factors in assessment of tubulointerstitial fibrosis in chronic glomerulonephritis]. *Ter Arkh.* 2007;79(6):10-15.

**18.** Honkanen EO, Teppo AM, Gronhagen-Riska C. Decreased urinary excretion of vascular endothelial growth factor in idiopathic membranous glomerulonephritis. *Kidney Int.* Jun 2000;57(6):2343-2349.

**19.** Scaglione R, Argano C, Corrao S, Di Chiara T, Licata A, Licata G. Transforming growth factor beta1 and additional renoprotective effect of combination ACE inhibitor and angiotensin II receptor blocker in hypertensive subjects with minor renal abnormalities: a 24-week randomized controlled trial. *J Hypertens.* Mar 2005;23(3):657-664.

**20.** Tam FW, Sanders JS, George A, et al. Urinary monocyte chemoattractant protein-1 (MCP-1) is a marker of active renal vasculitis. *Nephrol Dial Transplant.* Nov 2004;19(11):2761-2768.
**21.** Westhoff JH, Schildhorn C, Jacobi C, et al. Telomere shortening reduces regenerative capacity after acute kidney injury. *J Am Soc Nephrol.* Feb 2010;21(2):327-336.
**22.** Perico N, Remuzzi G, Benigni A. Aging and the kidney. *Curr Opin Nephrol Hypertens.* May 2011;20(3):312-317.
**23.** Schmitt R, Coca S, Kanbay M, Tinetti ME, Cantley LG, Parikh CR. Recovery of kidney function after acute kidney injury in the elderly: a systematic review and meta-analysis. *Am J Kidney Dis.* Aug 2008;52(2):262-271.
**24.** de Fijter JW, Mallat MJ, Doxiadis, II, et al. Increased immunogenicity and cause of graft loss of old donor kidneys. *J Am Soc Nephrol.* Jul 2001;12(7):1538-1546.
**25.** Ferlicot S, Durrbach A, Ba N, Desvaux D, Bedossa P, Paradis V. The role of replicative senescence in chronic allograft nephropathy. *Hum Pathol.* Sep 2003;34(9):924-928.
**26.** Melk A, Schmidt BM, Vongwiwatana A, Rayner DC, Halloran PF. Increased expression of senescence-associated cell cycle inhibitor p16INK4a in deteriorating renal transplants and diseased native kidney. *Am J Transplant.* Jun 2005;5(6):1375-1382.
**27.** Melk A, Schmidt BM, Takeuchi O, Sawitzki B, Rayner DC, Halloran PF. Expression of p16INK4a and other cell cycle regulator and senescence associated genes in aging human kidney. *Kidney Int.* Feb 2004;65(2):510-520.
**28.** Yang H, Fogo AB. Cell senescence in the aging kidney. *J Am Soc Nephrol.* Sep 2010;21(9):1436-1439.
**29.** Melk A, Kittikowit W, Sandhu I, et al. Cell senescence in rat kidneys in vivo increases with growth and age despite lack of telomere shortening. *Kidney Int.* Jun 2003;63(6):2134-2143.
**30.** Schmitt R, Cantley LG. The impact of aging on kidney repair. *Am J Physiol Renal Physiol.* Jun 2008;294(6):F1265-1272.
**31.** Jiang H, Ju Z, Rudolph KL. Telomere shortening and ageing. *Z Gerontol Geriatr.* Oct 2007;40(5):314-324.
**32.** Houben JM, Moonen HJ, van Schooten FJ, Hageman GJ. Telomere length assessment: biomarker of chronic oxidative stress? *Free Radic Biol Med.* Feb 1 2008;44(3):235-246.
**33.** Feng X, Wang L, Li Y. Change of telomere length in angiotensin II-induced human glomerular mesangial cell senescence and the protective role of losartan. *Mol Med Report.* Mar-Apr 2011;4(2):255-260.
**34.** Furumoto K, Inoue E, Nagao N, Hiyama E, Miwa N. Age-dependent telomere shortening is slowed down by enrichment of intracellular vitamin C via suppression of oxidative stress. *Life Sci.* 1998;63(11):935-948.
**35.** Kashino G, Kodama S, Nakayama Y, et al. Relief of oxidative stress by ascorbic acid delays cellular senescence of normal human and Werner syndrome fibroblast cells. *Free Radic Biol Med.* Aug 15 2003;35(4):438-443.
**36.** Melk A, Ramassar V, Helms LM, et al. Telomere shortening in kidneys with age. *J Am Soc Nephrol.* Mar 2000;11(3):444-453.
**37.** Tchakmakjian L, Gardner JP, Wilson PD, et al. Age-dependent telomere attrition as a potential indicator of racial differences in renal growth patterns. *Nephron Exp Nephrol.* 2004;98(3):e82-88.
**38.** Hamet P, Thorin-Trescases N, Moreau P, et al. Workshop: excess growth and apoptosis: is hypertension a case of accelerated aging of cardiovascular cells? *Hypertension.* Feb 2001;37(2 Part 2):760-766.
**39.** Tarry-Adkins JL, Ozanne SE, Norden A, Cherif H, Hales CN. Lower antioxidant capacity and elevated p53 and p21 may be a link between gender disparity in renal telomere shortening, albuminuria, and longevity. *Am J Physiol Renal Physiol.* Feb 2006;290(2):F509-516.
**40.** Hirota J, Usui R, Satoh T, Ikemoto S. Telomere position on the cat chromosome. *J Vet Med Sci.* Oct 1996;58(10):1025-1026.

**41.** McKevitt TP, Nasir L, Wallis CV, Argyle DJ. A cohort study of telomere and telomerase biology in cats. *Am J Vet Res.* Dec 2003;64(12):1496-1499.
**42.** Nakagawa S, Gemmell NJ, Burke T. Measuring vertebrate telomeres: applications and limitations. *Mol Ecol.* Sep 2004;13(9):2523-2533.
**43.** Meeker AK, Gage WR, Hicks JL, et al. Telomere length assessment in human archival tissues: combined telomere fluorescence in situ hybridization and immunostaining. *Am J Pathol.* Apr 2002;160(4):1259-1268.
**44.** Brandt LE, Bohn AA, Charles JB, Ehrhart EJ. Localization of Canine, Feline, and Mouse Renal Membrane Proteins. *Vet Pathol.* Jun 28 2011.
**45.** Reyes JL, Lamas M, Martin D, et al. The renal segmental distribution of claudins changes with development. *Kidney Int.* Aug 2002;62(2):476-487.
**46.** Roudebush P, Polzin DJ, Ross SJ, Towell TL, Adams LG, Dru Forrester S. Therapies for feline chronic kidney disease. What is the evidence? *J Feline Med Surg.* Mar 2009;11(3):195-210.
**47.** Chan DL. The Inappetent Hospitalised Cat: clinical approach to maximising nutritional support. *J Feline Med Surg.* Nov 2009;11(11):925-933.
**48.** Parker VJ, Freeman LM. Association between body condition and survival in dogs with acquired chronic kidney disease. *J Vet Intern Med.* Nov-Dec 2011;25(6):1306-1311.
**49.** Kopple JD. Effect of nutrition on morbidity and mortality in maintenance dialysis patients. *Am J Kidney Dis.* Dec 1994;24(6):1002-1009.
**50.** Laflamme DP. Nutrition for aging cats and dogs and the importance of body condition. *Vet Clin North Am Small Anim Pract.* May 2005;35(3):713-742.
**51.** Sparkes AH. Feeding old cats--an update on new nutritional therapies. *Top Companion Anim Med.* Feb 2011;26(1):37-42.
**52.** Michel KE. Management of anorexia in the cat. *J Feline Med Surg.* Mar 2001;3(1):3-8.
**53.** Elliott J, Rawlings JM, Markwell PJ, Barber PJ. Survival of cats with naturally occurring chronic renal failure: effect of dietary management. *J Small Anim Pract.* Jun 2000;41(6):235-242.
**54.** Plantinga EA, Everts H, Kastelein AM, Beynen AC. Retrospective study of the survival of cats with acquired chronic renal insufficiency offered different commercial diets. *Vet Rec.* Aug 13 2005;157(7):185-187.
**55.** Ross SJ, Osborne CA, Kirk CA, Lowry SR, Koehler LA, Polzin DJ. Clinical evaluation of dietary modification for treatment of spontaneous chronic kidney disease in cats. *J Am Vet Med Assoc.* Sep 15 2006;229(6):949-957.
**56.** Ljutic D, Perkovic D, Rumboldt Z, Bagatin J, Hozo I, Pivac N. Comparison of ondansetron with metoclopramide in the symptomatic relief of uremia-induced nausea and vomiting. *Kidney Blood Press Res.* 2002;25(1):61-64.
**57.** Pae CU. Low-dose mirtazapine may be successful treatment option for severe nausea and vomiting. *Prog Neuropsychopharmacol Biol Psychiatry.* Aug 30 2006;30(6):1143-1145.
**58.** Kast RE, Foley KF. Cancer chemotherapy and cachexia: mirtazapine and olanzapine are 5-HT3 antagonists with good antinausea effects. *Eur J Cancer Care (Engl).* Jul 2007;16(4):351-354.
**59.** Chang FL, Ho ST, Sheen MJ. Efficacy of mirtazapine in preventing intrathecal morphine-induced nausea and vomiting after orthopaedic surgery*. *Anaesthesia.* Dec 2010;65(12):1206-1211.
**60.** Chen CC, Lin CS, Ko YP, Hung YC, Lao HC, Hsu YW. Premedication with mirtazapine reduces preoperative anxiety and postoperative nausea and vomiting. *Anesth Analg.* Jan 2008;106(1):109-113, table of contents.
**61.** Center SA, Elston TH, Rowland PH, et al. Fulminant hepatic failure associated with oral administration of diazepam in 11 cats. *J Am Vet Med Assoc.* Aug 1 1996;209(3):618-625.
**62.** de Boer T. The pharmacologic profile of mirtazapine. *J Clin Psychiatry.* 1996;57 Suppl 4:19-25.
**63.** Timmer CJ, Sitsen JM, Delbressine LP. Clinical pharmacokinetics of mirtazapine. *Clin Pharmacokinet.* Jun 2000;38(6):461-474.
**64.** Riechelmann RP, Burman D, Tannock IF, Rodin G, Zimmermann C. Phase II trial of mirtazapine for cancer-related cachexia and anorexia. *Am J Hosp Palliat Care.* Mar 2010;27(2):106-110.

**65.** Cahill C. Mirtazapine as an antiemetic. *Veterinary Forum.* 2006:34-36.
**66.** Reinders ME, Fibbe WE, Rabelink TJ. Multipotent mesenchymal stromal cell therapy in renal disease and kidney transplantation. *Nephrol Dial Transplant.* Jan 2010;25(1):17-24.
**67.** Martinello T, Bronzini I, Maccatrozzo L, et al. Canine adipose-derived-mesenchymal stem cells do not lose stem features after a long-term cryopreservation. *Research in veterinary science.* Aug 2011;91(1):18-24.
**68.** Schaffler A, Buchler C. Concise review: adipose tissue-derived stromal cells--basic and clinical implications for novel cell-based therapies. *Stem cells.* Apr 2007;25(4):818-827.
**69.** Semedo P, Correa-Costa M, Antonio Cenedeze M, et al. Mesenchymal stem cells attenuate renal fibrosis through immune modulation and remodeling properties in a rat remnant kidney model. *Stem Cells.* Dec 2009;27(12):3063-3073.
**70.** Cavaglieri RC, Martini D, Sogayar MC, Noronha IL. Mesenchymal stem cells delivered at the subcapsule of the kidney ameliorate renal disease in the rat remnant kidney model. *Transplant Proc.* Apr 2009;41(3):947-951.
**71.** Martin DR, Cox NR, Hathcock TL, Niemeyer GP, Baker HJ. Isolation and characterization of multipotential mesenchymal stem cells from feline bone marrow. *Exp Hematol.* Aug 2002;30(8):879-886.
**72.** Togel F, Cohen A, Zhang P, Yang Y, Hu Z, Westenfelder C. Autologous and allogeneic marrow stromal cells are safe and effective for the treatment of acute kidney injury. *Stem cells and development.* Apr 2009;18(3):475-485.
**73.** Togel F, Weiss K, Yang Y, Hu Z, Zhang P, Westenfelder C. Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. *Am J Physiol Renal Physiol.* May 2007;292(5):F1626-1635.
**74.** Barry FP, Murphy JM, English K, Mahon BP. Immunogenicity of adult mesenchymal stem cells: lessons from the fetal allograft. *Stem cells and development.* Jun 2005;14(3):252-265.
**75.** McTaggart SJ, Atkinson K. Mesenchymal stem cells: immunobiology and therapeutic potential in kidney disease. *Nephrology.* Feb 2007;12(1):44-52.
**76.** Semedo P, Wang PM, Andreucci TH, et al. Mesenchymal stem cells ameliorate tissue damages triggered by renal ischemia and reperfusion injury. *Transplantation proceedings.* Mar 2007;39(2):421-423.
**77.** Morigi M, Imberti B, Zoja C, et al. Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure. *J Am Soc Nephrol.* Jul 2004;15(7):1794-1804.
**78.** Little MH, Rae FK. Review article: Potential cellular therapies for renal disease: can we translate results from animal studies to the human condition? *Nephrology (Carlton).* Sep 2009;14(6):544-553.
**79.** Kim SS, Park HJ, Han J, et al. Improvement of kidney failure with fetal kidney precursor cell transplantation. *Transplantation.* May 15 2007;83(9):1249-1258.
**80.** Kitamura S, Yamasaki Y, Kinomura M, et al. Establishment and characterization of renal progenitor like cells from S3 segment of nephron in rat adult kidney. *Faseb J.* Nov 2005;19(13):1789-1797.
**81.** Togel F, Yang Y, Zhang P, Hu Z, Westenfelder C. Bioluminescence imaging to monitor the in vivo distribution of administered mesenchymal stem cells in acute kidney injury. *Am J Physiol Renal Physiol.* Jul 2008;295(1):F315-321.
**82.** Lee SR, Lee SH, Moon JY, et al. Repeated administration of bone marrow-derived mesenchymal stem cells improved the protective effects on a remnant kidney model. *Ren Fail.* 2010;32(7):840-848.
**83.** Villanueva S, Ewertz E, Carrion F, et al. Mesenchymal stem cell injection ameliorates chronic renal failure in a rat model. *Clin Sci (Lond).* Dec 2011;121(11):489-499.
**84.** Kirpatovskii VI, Kazachenko AV, Plotnikov EY, et al. Functional aftereffects of intraparenchymatous injection of human fetal stem and progenitor cells to rats with chronic and acute renal failure. *Bull Exp Biol Med.* Apr 2006;141(4):500-506.

**85.** Choi S, Park M, Kim J, Hwang S, Park S, Lee Y. The role of mesenchymal stem cells in the functional improvement of chronic renal failure. *Stem Cells Dev.* Apr 2009;18(3):521-529.
**86.** Ninichuk V, Gross O, Segerer S, et al. Multipotent mesenchymal stem cells reduce interstitial fibrosis but do not delay progression of chronic kidney disease in collagen4A3-deficient mice. *Kidney Int.* Jul 2006;70(1):121-129.

# CHAPTER 2: RESEARCH OVERVIEW AND SPECIFIC AIMS

## 2.1 Research Overview

Chronic kidney disease is one of the leading causes of morbidity and mortality in geriatric cats, affecting conservatively 30% of the population; an estimated 24 million cats nationwide. Despite the common nature of the disease, its etiology is yet unknown, and there is no definitive cure short of renal transplantation. The goals of the research described in this dissertation were to explore possible etiologies of kidney disease and to develop novel treatment strategies to help cats afflicted with this disease. Chapter 3 focuses on the assessment of telomere length and cellular senescence in CKD, which may provide some insight into pathogenesis of aging in the kidney and its propensity for disease, as well as provide a foundation for new treatment strategies. Chapter 4 describes studies on the pharmacokinetics and pharmacodynamics of mirtazapine, a novel appetite stimulant, which will potentially improve the nutrition and quality of life of cats with CKD. Chapter 5 investigates the use of mesenchymal stem cell therapy as a novel treatment for feline CKD.

## 2.2 Specific Aim 1 (Chapter 3: Telomeres)

Telomeres are protective structures at the ends of chromosomes that gradually shorten with age, and eventually reach a critically shortened length that causes cells to stop dividing, a state called senescence. A leading hypothesis of aging suggests that as cellular senescence increases, organ function declines. We are investigating the role of cellular senescence as an underlying contributor to feline CKD. There is preliminary information from other investigators that telomeres in cats do indeed shorten with increasing age,[1] thus implicating telomere shortening in the feline aging process. However, no attempt has been made to correlate telomere length or other markers of cellular senescence with any disease process in cats. Shortened telomeres and increased senescence have been associated with kidney disease in humans and may be related to the susceptibility of the aging kidney to various insults.[2,3] The specific aim for this chapter was to investigate the role of telomere length and cellular senescence in feline CKD. The **hypothesis for specific aim 1 is that cats with CKD will have shortened telomeres and increased cellular senescence when compared to young normal cats and geriatric normal cats**. A novel diagnostic method, combined telomere fluorescence *in situ* hybridization and immunostaining (TELI-FISH),[4] was optimized for feline paraffin-embedded tissues to facilitate telomere measurement in specific cell populations within the kidney. Demonstrating a correlation between CKD, telomere shortening and cellular senescence could potentially create new treatment strategies for this common disease.

## 2.3 Specific Aim 2 (Chapter 4: Mirtazapine)

There currently is no definitive cure for feline CKD other than renal transplantation, which is not feasible for many owners. Therefore the mainstay of treatment is medical management. Inappetence is one of the clinical signs that owners struggle with and often is most concerning to them from a quality of life standpoint as it is more tangible than metabolic disturbances. Several recent studies have documented the therapeutic value of specially formulated diets in the management of CKD.[5-7] The failure of the patient to eat the diet negates the benefit of dietary management, and therefore a key therapeutic target for these patients is the maintenance of appetite and food intake. New pharmacological options for the management of inappetence in CKD cats would be of great interest to the veterinary community. Mirtazapine is an appetite stimulant and anti-nausea medication that has recently gained popularity in veterinary medicine and anecdotally appears to be helpful for the management of appetite. However, no pharmacokinetic or pharmacodynamic information exists on the drug in cats.

The focus of this chapter is the assessment of the pharmacokinetics and pharmacodynamics of commonly prescribed doses of mirtazapine in normal cats, geriatric cats and cats with CKD, to provide information to further guide the use of mirtazapine as an appetite stimulant in cats, specifically for those suffering from CKD. This information could potentially result in decreased incidence of unwanted drug side effects and improved body condition and quality of life for cats with CKD. **The hypothesis for specific aim 2 is that mirtazapine is clinically useful for the management of feline CKD and results in increased appetite and weight gain.** In order to test this hypothesis, pharmacokinetic studies were performed in young normal cats, geriatric normal cats and cats with CKD to allow optimization of drug administration. A placebo-controlled blinded crossover clinical trial was then performed in cats with CKD.

## 2.4 Specific Aim 3 (Chapter 5: Stem Cells)

CKD is characterized by tubulointerstitial damage, fibrosis and progressive loss of renal function, and is commonly described as the final common pathway after any one of multiple types of renal insults. Regardless of the initial insult, once a threshold of renal damage has been reached, progression is irreversible and appears consistent in character. Mesenchymal stem cell (MSC) therapy has the potential to improve or stabilize renal function in animals with renal failure, based on evidence from rodent model studies of induced renal disease. In several rodent CKD models, administration of MSC has been beneficial, especially with respect to reducing intra-renal inflammation and suppressing fibrosis.[8-11] *In vitro* studies have demonstrated that MSC can produce growth factors, cytokines, and anti-inflammatory mediators, all of which could help maintain or improve renal function and suppress intra-renal inflammation.[12-14] At present, there is little published work regarding the use of MSC for treatment of naturally occurring CKD.

This chapter focuses on the evaluation of MSC therapy as a novel treatment strategy for cats with CKD. **The hypothesis for specific aim 3 is that injection of MSC will not be harmful and will improve renal function in CKD cats due to anti-inflammatory effects.** A series of pilot studies was used to test this hypothesis; a pilot study of intrarenal injection of autologous stem cells and two pilot studies of intravenously injected allogeneic cryopreserved MSC. Minimum database, GFR and urinary cytokines were measured in clinical trial patients to assess intra-renal inflammation, fibrosis and vascular health and the possible effects of MSC injection on these factors.

## 2.5 References


**1.** McKevitt TP, Nasir L, Wallis CV, Argyle DJ. A cohort study of telomere and telomerase biology in cats. *Am J Vet Res.* Dec 2003;64(12):1496-1499.

**2.** Melk A, Schmidt BM, Vongwiwatana A, Rayner DC, Halloran PF. Increased expression of senescence-associated cell cycle inhibitor p16INK4a in deteriorating renal transplants and diseased native kidney. *Am J Transplant.* Jun 2005;5(6):1375-1382.

**3.** Schmitt R, Cantley LG. The impact of aging on kidney repair. *Am J Physiol Renal Physiol.* Jun 2008;294(6):F1265-1272.

**4.** Meeker AK, Gage WR, Hicks JL, et al. Telomere length assessment in human archival tissues: combined telomere fluorescence in situ hybridization and immunostaining. *Am J Pathol.* Apr 2002;160(4):1259-1268.

**5.** Elliott J, Rawlings JM, Markwell PJ, Barber PJ. Survival of cats with naturally occurring chronic renal failure: effect of dietary management. *J Small Anim Pract.* Jun 2000;41(6):235-242.

**6.** Plantinga EA, Everts H, Kastelein AM, Beynen AC. Retrospective study of the survival of cats with acquired chronic renal insufficiency offered different commercial diets. *Vet Rec.* Aug 13 2005;157(7):185-187.

**7.** Ross SJ, Osborne CA, Kirk CA, Lowry SR, Koehler LA, Polzin DJ. Clinical evaluation of dietary modification for treatment of spontaneous chronic kidney disease in cats. *J Am Vet Med Assoc.* Sep 15 2006;229(6):949-957.

**8.** Villanueva S, Ewertz E, Carrion F, et al. Mesenchymal stem cell injection ameliorates chronic renal failure in a rat model. *Clin Sci (Lond).* Dec 2011;121(11):489-499.

**9.** Semedo P, Correa-Costa M, Antonio Cenedeze M, et al. Mesenchymal stem cells attenuate renal fibrosis through immune modulation and remodeling properties in a rat remnant kidney model. *Stem Cells.* Dec 2009;27(12):3063-3073.

**10.** Ninichuk V, Gross O, Segerer S, et al. Multipotent mesenchymal stem cells reduce interstitial fibrosis but do not delay progression of chronic kidney disease in collagen4A3-deficient mice. *Kidney Int.* Jul 2006;70(1):121-129.

**11.** Lee SR, Lee SH, Moon JY, et al. Repeated administration of bone marrow-derived mesenchymal stem cells improved the protective effects on a remnant kidney model. *Ren Fail.* 2010;32(7):840-848.

**12.** McTaggart SJ, Atkinson K. Mesenchymal stem cells: immunobiology and therapeutic potential in kidney disease. *Nephrology.* Feb 2007;12(1):44-52.

**13.** Barry FP, Murphy JM, English K, Mahon BP. Immunogenicity of adult mesenchymal stem cells: lessons from the fetal allograft. *Stem cells and development.* Jun 2005;14(3):252-265.

**14.** Togel F, Weiss K, Yang Y, Hu Z, Zhang P, Westenfelder C. Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. *Am J Physiol Renal Physiol.* May 2007;292(5):F1626-1635.

# CHAPTER 3: ASSESSMENT OF TELOMERE LENGTH AND CELLULAR SENESCENCE IN FELINE CHRONIC KIDNEY DISEASE

I would like to acknowledge the contributions of Drs. Susan Bailey for providing the intellectual question and grant support for this project, David Maranon for collaboration in developing the Telomere FISH assay, Alan Meeker for advice regarding the TELI-FISH assay, Shannon McLeland for assessment of histopathologic samples, as well as Brad Charles for assistance with IHC and image analysis and Willy Brock for assistance with β-galactosidase assays.

## 3.1 Abstract

**Background:** Telomeres are protective structures at the ends of chromosomes that gradually shorten with age, and eventually reach a critically shortened length that triggers a permanent cell cycle arrest called cellular senescence.

**Aim:** The aim of this study was to determine if shortened telomere length and cellular senescence are associated with chronic kidney disease (CKD) in cats.

**Methods:** Kidney, liver and skin samples from 12 CKD cats, 12 young normal cats and 6 normal geriatric cats were analyzed. Standard telomere *in situ* fluorescent hybridization (TEL-FISH) was combined with immunohistochemistry (TELI-FISH) in kidney samples to identify two specific populations of cells in the renal cortex for analysis of telomere length: proximal tubular epithelial cells and distal tubular epithelial cells. TEL-FISH was also performed on liver and skin samples. β-galactosidase assay to evaluate cellular senescence was performed on kidney and liver tissues.

**Results:** There was a statistically significant decrease in the average telomere fluorescence intensity (TFI) of proximal tubular epithelial cells of CKD cats in comparison to young normal and geriatric normal cats. There was a statistically significant decrease in the average TFI in the distal tubular epithelial cells of

CKD cats in comparison to young normal cats. No difference in average TFI was found between young normal and geriatric normal cats for either cell type. No difference in average TFI was found for liver or skin samples from any group. When histograms of individual TFI were compared, there was a statistically significant decrease in the TFI of proximal tubular epithelial cells and distal tubular epithelial cells of CKD cats in comparison to young normal and geriatric normal cats. A statistically significant increase in β-galactosidase staining was seen in CKD kidney samples in comparison to young normal cats. CKD cats tended to have increased β-galactosidase staining in comparison to normal geriatric cats, but this did not reach statistical significance. Minimal β-galactosidase staining was seen in liver samples from all three groups, however, the CKD cats had a statistically significant increase in staining compared to other groups.

**Conclusions:** Significantly shortened telomeres and increased cellular senescence were observed in the kidneys of CKD cats in comparison to young normal and geriatric normal cats.

## 3.2 Introduction

Chronic kidney disease (CKD) is a common medical condition in elderly cats, yet an etiology for this disease process has yet to be elucidated. In humans there is progressive loss of renal parenchyma and function with age.[1] The phenotype of renal aging is described not only as the loss of function and renal mass, but as the loss of an appropriate response toward injury. In aging human populations kidney injury is more common, clinically more severe, and kidney function is less likely to recover.[2] This is particularly important in renal transplant medicine where age of the donor is the most important predictor of long term success; older donor kidneys are more likely to fail even when rejection or other insults are mild in nature.[3,4] Whether CKD in cats results from a similar aging process that predisposes the organ to failure or if other factors are involved remains to be determined.

Telomeres are protective structures at the ends of chromosomes that are critical for chromosome stability and effective cellular replication and repair. They cap the chromosome and prevent the end from being identified as a DNA double-strand break (DSB), thereby initiating a DNA damage response

resulting in cell cycle arrest, apoptosis, or death.[5] Telomeres shorten with each replication cycle due to "end replication problem"; conventional DNA polymerases cannot fully replicate to the very end of a DNA strand and so with each cycle some telomeric DNA is lost. Additional factors can also lead to telomere shortening, such as deletions, and oxidative stress.[5] Telomerase is a specialized reverse transcriptase that can compensate by creating new telomeres *de novo*, but it is generally not active in somatic cells at sufficient levels to maintain telomere length after embryogenesis. However, telomerase is often reactivated in cancer.[5]

When telomeres reach a critically shortened state, they become dysfunctional, unable to provide end protection, and so themselves are detected as DNA damage and trigger a response resulting in permanent cell cycle arrest, and the cell becomes senescent.[5] Most human tissues have been documented to experience telomere shortening with age, and telomeres have been shown to be shortened in several disease states.[6] Increased cellular senescence has been documented in human renal disease conditions.[1,7-9] Telomeres are acutely sensitive to oxidative stress, in part due to their high content of guanine residues but also because reactive oxygen species (ROS) produce single strand breaks and telomeric DNA is thought to be deficient in the repair of single strand breaks.[6] There also appears to be an association between inflammation, oxidative stress and telomere shortening thus investigation of telomere length is particularly pertinent in any inflammatory disease condition.[6]

The purpose of this study was to assess telomere length using a novel approach, TELI-FISH, which was adapted for use in cats to allow analysis of specific populations of cells within the renal cortex, specifically proximal tubular segments and distal tubular segments of the nephron.[10] TELI-FISH has advantages over traditional telomere length analysis techniques in that it combines IHC with telomere FISH to allow identification of the specific cellular compartment of interest, i.e. the proximal tubule, and to avoid other cells that may be present , i.e. inflammatory infiltrates.[10] As feline CKD is characterized by tubulointerstitial inflammation,[11] and oxidative stress has been documented in these patients,[12] we hypothesized that shortened telomeres and increased cellular senescence would be present in cats affected

by CKD. Demonstration of an association between telomere shortening, cellular senescence and feline CKD has the potential to present novel treatment strategies.

## 3.3 Materials and Methods

### *3.3.1 Sample Collection*

Samples were collected from 12 cats with CKD, 12 young normal cats and 6 geriatric cats without evidence of CKD. Kidney, liver and skin samples were collected in formalin for histopathology and TELI-FISH analysis as well as quick frozen in OCT media for B-galactosidase analysis. CKD was defined as a cat with a clinical pathology history of a urine specific gravity <1.035, and a creatinine over 2.0. Generally CKD patients included in the study were long term clients at Colorado State University and their clinical history was well known and had been followed for months to years. Young normal cats were acquired from local humane societies where they were euthanized as unwanted pets. Although in some cases a clinical history was not available, young normal cats were defined by physical exam findings of healthy body condition and minimal dental tartar, and a normal complete blood count, chemistry and urinalysis (urine specific gravity >1.035). Blood and urine samples were collected immediately post-euthanasia, the former via cardiac stick, the latter via cystocentesis. Cats with significant hemolysis index were not included in the study. Normal geriatric cats were defined as those with no clinical history of CKD, a urine specific gravity >1.035, and minimal changes on renal histopathology. Some samples were from clinical patients at Colorado State and some were acquired from local humane societies similar to the young normal cats.

### *3.3.2 Histopathologic Assessment*

Dr. Shannon McLeland, a pathology resident, assessed kidney samples included in this study to confirm that they were appropriately categorized; and liver samples to confirm that they were histopathologically normal.

### *3.3.3. FISH Slide Preparation for Analysis*

*Deparaffinization (kidney, liver and skin):*

Kidney and liver samples were cut from paraffin blocks at a thickness of 2 to 2.5 microns. Slides were placed in a warming chamber at 65° C for 10 minutes to melt paraffin, followed by three xylene washes of 10 minutes to remove paraffin. Slides were hydrated through a graded ethanol series, 3 minutes in each (100%, 95% and 75%), and rinsed in phosphate buffered saline (PBS). Slides were then immersed in 3% hydrogen peroxide for 5 minutes and washed in PBS. Slides were immersed in methanol for 5 minutes. Kidney samples that were undergoing antigen retrieval before TELI-FISH were then immersed in 3% paraformaldehyde for 10 minutes.

*Antigen Retrieval (kidney):*

For kidney samples undergoing TELI-FISH, antigen retrieval was performed by placing slides in a plastic slide holder, immersing in citrate buffer (Dako target unmasking solution, Carpinteria, CA) and steamed at 125°C for 1 minute in a pressure cooker. Slides were gradually cooled, and rinsed with deionized (DI) water. Slides were placed in buffer for transport and then dehydrated through a graded ethanol series, 2 minutes in each (75, 95, 100%) and stored at 4°C until TELI-FISH analysis.

*TEL-FISH (liver and skin):*

Slides were placed in DI water for 3 minutes and then immersed for 60 seconds in DI water with 1% Tween-20 detergent (Sigma Aldrich, St. Louis, MO). Slides were dipped briefly in DI to wash off Tween. 100 µl of 100mcg/ml proteinase k (Roche, San Francisco, CA) was applied to slides, coverslipped and incubated at 37° C for 15 minutes. Slides were washed in PBS for 2 minutes and dehydrated through a graded ethanol series, 2 minutes in each (75, 95, 100%) and air dried. A peptide nucleic acid (PNA) telomere probe was utilized for hybridization (G-rich probe, Cy3 labeled; red, Biosynthesis, Lewisville, TX) and was prepared by diluting 5 µl probe in 36 µl formamide (Sigma Aldrich, St. Louis, MO), 12 µl TRIS buffer, 2.5 µl KCl (Sigma Aldrich, St. Louis, MO) and 0.6 µl MgCl (Sigma Aldrich, St. Louis,

MO). Fifty µl of probe was applied to the slide, coverslipped and denatured at 85° C for 5 minutes. Slides were then incubated for 2 hours in a hydrated slide box at 37° C. After incubation slides were washed in a series of washes for 2.5 minutes each at 43.5° C. The composition of the washes were: wash 1 and 2: 50% formamide in 2X sodium citrate (SSC), wash 3 and 4: 2X SSC, and wash 5 and 6: 2X SSC + 0.1% NP40. Fifty µl DAPI (in Prolong Gold Antifade, Invitrogen, Carlsbad, CA) was applied to each slide and coverslipped. Slides were stored at -20° C before image analysis.

*TELI-FISH (kidney):*

Slide preparation was as previously described except that after the telomeric probe was hybridized, the slides were incubated sequentially with two primary antibodies, aquaporin 1(mouse anti-Aquaporin 1, Millipore, Billerica, MA) to identify proximal tubule segments and cytokeratin AE1/AE3 (rabbit anti-cytokeratin, Cell Marque, Rocklin, CA) to identify distal proximal tubule segments.[13,14] Both primary antibodies were incubated for 2 hours at room temperature at a concentration of 1:300. As secondary antibodies, an anti-mouse-Alexa 488 and an anti-rabbit-Alexa 647 (Invitrogen, Carlsbad, CA) were applied to visualize aquaporin 1 (green) and cytokeratin (red). The concentration of these antibodies was 1:750, incubated for 30 minutes at room temperature. After each incubation, slides were washed in sequential washes 3-6 (2.5 minutes in each wash) at 43.5° C.

### *3.3.4. FISH Image Capture and Processing*

Image Z stacks were taken using a Nikon Eclipse 600 microscope with a 100x objective, capturing fluorescent images with a Coolsnap ES camera using Metamorph software (Molecular Devices, Sunnyvale, CA). For each cell population (renal proximal tubule, renal distal tubule, liver and skin) 15 to 20 images were obtained by taking twenty-six stacks of 0.2 um per plane in 2 different wavelengths (Dapi and Cy3). For renal tubular images, one additional 2D image was taken for each cell type as a cell type identification reference (using FITC for aquaporin positive proximal tubules and Cy5 for cytokeratin positive distal tubules). After acquisition of the stacks, 3D-deconvolution was performed to obtain a

higher resolution of telomere signals. The final image was the maximum projection of the 26 stacks which allowed analysis of all telomere signals present in the whole extension of each cell nuclei.

### *3.3.5. FISH Data Analysis with TELOMETER*

Analysis of TFI was performed using TELOMETER, an Image J plug in program available as a download from http://demarzolab.pathology.jhmi.edu/telometer/downloads/index.html. Telomere signals from 60 nuclei per sample were analyzed using custom program settings (minimum object size: 1; maximum object size: 350; despeckle ratio: 0.3, rolling ball size: 1). Telomere fluorescence measurements were imported into Excel and mean cell TFI as well as mean sample TFI were calculated. Individual TFI frequency histograms incorporating all values measured were also created for each sample group using Prism software (Prism 5, GraphPad, La Jolla, CA).

### *3.3.6. FISH Statistical Analysis*

Mean sample TFI for kidney and liver from all three groups of cats were statistically compared using one way ANOVA with Dunn's post hoc analysis in Prism 5 software (Prism GraphPad, La Jolla, CA). Frequency histograms were created using Prism software. Finite mixed model analysis was performed on individual TFI histograms using SAS software (SAS 9.3, SAS Institute Inc, Cary, NC).

### *3.3.7. β-galactosidase Slide Preparation*

Kidney and liver samples were initially collected in optimal cutting temperature (OCT) media (Sakura Finetek USA, Torrance, CA) at the time of death and stored at -80° C until analysis. OCT blocks were cryosectioned at 4 microns (Leica CM 1850, Leica Microsystems, Wetzlar, Germany) and stored at -20° C until analysis. β-galactosidase analysis was performed using a commercially available kit for assessment of cellular senescence (#9860, Cell Signaling Technology Inc., Danvers, MA) which is based on the commonly utilized Dimri protocol.[15] β-galactosidase was prepared fresh for each sample run precisely as described in the kit, with the additional step of adding 4 microliters of HCL to the final

solution to result in a pH of 6.0. 200 µl of solution was applied to each slide, coverslipped, sealed with rubber cement and incubated for 14 hours at 37° C. Coverslips were removed; slides were rinsed in DI and counterstained with eosin, dehydrated and mounted with xylene mounting media before analysis.

### *3.3.8. β-galactosidase Staining Combined with Aquaporin 1 Immunohistochemistry*

Select samples (1/3 of CKD sample set) were co-stained with β-galactosidase and aquaporin 1 immunohistochemistry in order to confirm proximal tubules were β-galactosidase stain positive. Slides were prepared for β-galactosidase staining as described above, but prior to counterstain and mounting, immunohistochemistry was performed. Slides were placed in a coverplate system and washed with tris-buffered saline (TBS) for 5 minutes. A casein block (Biocare Sniper, Biocare Medical, Concord, CA) was applied for 10 minutes, followed by a 5 minute TBS wash and incubation with the aquaporin 1 primary antibody for 16 hours at 4°C. Slides were then washed with TBS for 5 minutes, incubated with 3% hydrogen peroxide for 15 minutes, washed three times with TBS for 5 minutes, and incubated with a secondary antibody (Envision+, Dako, Carpinteria, CA) for 30 minutes. Slides were washed three times with TBS for 5 minutes, and DAB chromagen (Biocare Medical, Concord, CA ) was applied for 5 minutes followed by a 5 minute TBS wash. Slides were counterstained with eosin, dehydrated and mounted with xylene mounting media.

### *3.3.9. β-galactosidase Image Capture and Analysis*

Image capture of β-galactosidase samples was performed using a Zeiss microscope with a 20x objective, and using a Coolsnap ES camera. Four images of the renal cortex or liver parenchyma were obtained from each sample. Image analysis was performed using AxioVision software (Carl Zeiss MicroImaging, Jena, Germany). Statistical analysis was performed using a one way ANOVA with Dunn's post hoc analysis in Prism 5 software.

## 3.4 Results

### *3.4.1 Sample Collection*

Tissues from 12 young apparently healthy cats were collected. Of these cats 11 were domestic shorthairs (DSH) and 1 was a domestic longhair. Three cats were neutered males, 2 were intact males, 5 were neutered females and 1 was an intact female. The mean age was 2.7 years (range 0.8 – 4 years). Tissues from 12 cats with CKD were collected. Of these cats 8 were DSH, 3 were Siamese crosses and 1 was a Ragdoll. There were 4 neutered males and 8 neutered females. The mean age was 14.8 years (range 6-19 years). Tissues from 6 geriatric cats without CKD were collected. The study goal was 12 cats in this group, but elderly cats without renal compromise were very difficult to find. Of these cats 4 were neutered females and 2 were neutered males and all 6 cats were DSH. The mean age was 11.5 years (range 10 -16).

### *3.4.2 Histopathologic Assessment*

Sections of kidneys consisted of cortex, medulla and renal pelvis and were categorized into the following groups: normal, normal geriatric and CKD cats. Normal geriatric cats were determined based on either absence of histologic abnormalities or very mild, scattered, cortical interstitial mononuclear infiltrates with occasional tubular and glomerular basement membrane thickening. In contrast, typical CKD cats had severe interstitial infiltrates in conjunction with fibrosis, tubular loss, degeneration and glomerulosclerosis. Normal cats, in contrast, lacked such histologic changes. Sections of liver from study cats were assessed for the presence of metastatic or primary hepatic neoplasia, inflammation or hepatopathy. Sections of skin from study cats were assessed for the presence of neoplasia or dermatopathy. Patients were excluded based on the presence of inflammatory disease and or neoplasia. Only one potential case was excluded due to hepatopathy, all skin sections examined were histologically normal.

### *3.4.3. TELI-FISH Analysis*

Telomere fluorescence in situ hybridization was successfully combined with immunostaining (TELI-FISH) to identify two cell populations in the renal cortex for telomere length analysis. An aquaporin 1 antibody (green) was used to identify sections of proximal tubule (**Figure 3.1**) and a cytokeratin antibody (red) was used to identify distal tubule segments (**Figure 3.1**). Loss of proximal tubule segments was very apparent in CKD samples (**Figure 3.2**). Adequate telomere signal for analysis was achieved using this technique (**Figure 3.3**).

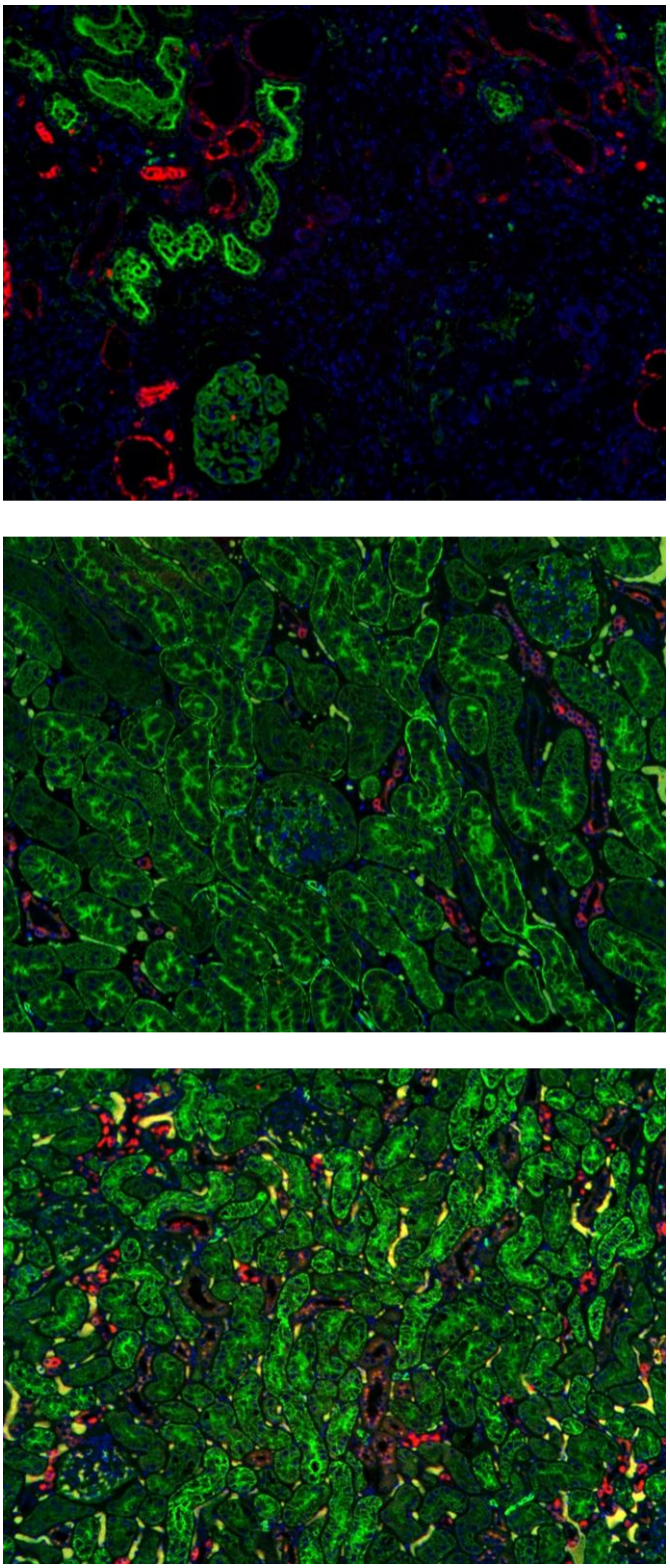


**Figure 3.1:** TELI-FISH analysis of renal cortex in a CKD cat (top), normal cat (middle) and normal geriatric cat (bottom). Aquaporin 1 positive proximal tubules are green, Cytokeratin positive distal tubules are red, nuclei are blue (10x). Extensive atubular areas with large numbers of nuclei exist in the CKD sample. Nuclei in these areas may represent inflammatory infiltrate and/or fibroblasts.

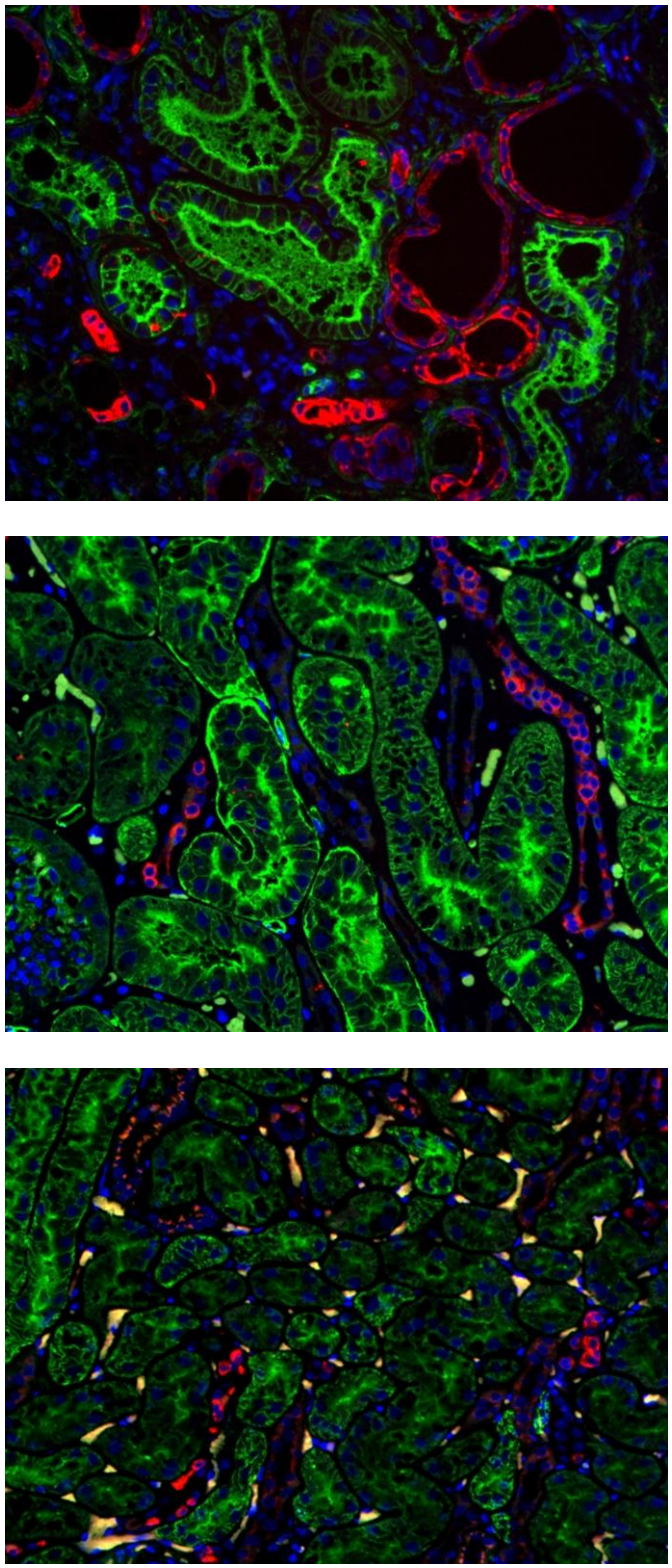


**Figure 3.2:** TELI-FISH analysis of renal cortex in a CKD cat (top), normal cat (middle) and normal geriatric cat (bottom). Aquaporin 1 positive proximal tubules are green, Cytokeratin positive distal tubules are red, nuclei are blue (20x). Loss of proximal tubules is apparent in the CKD cat.

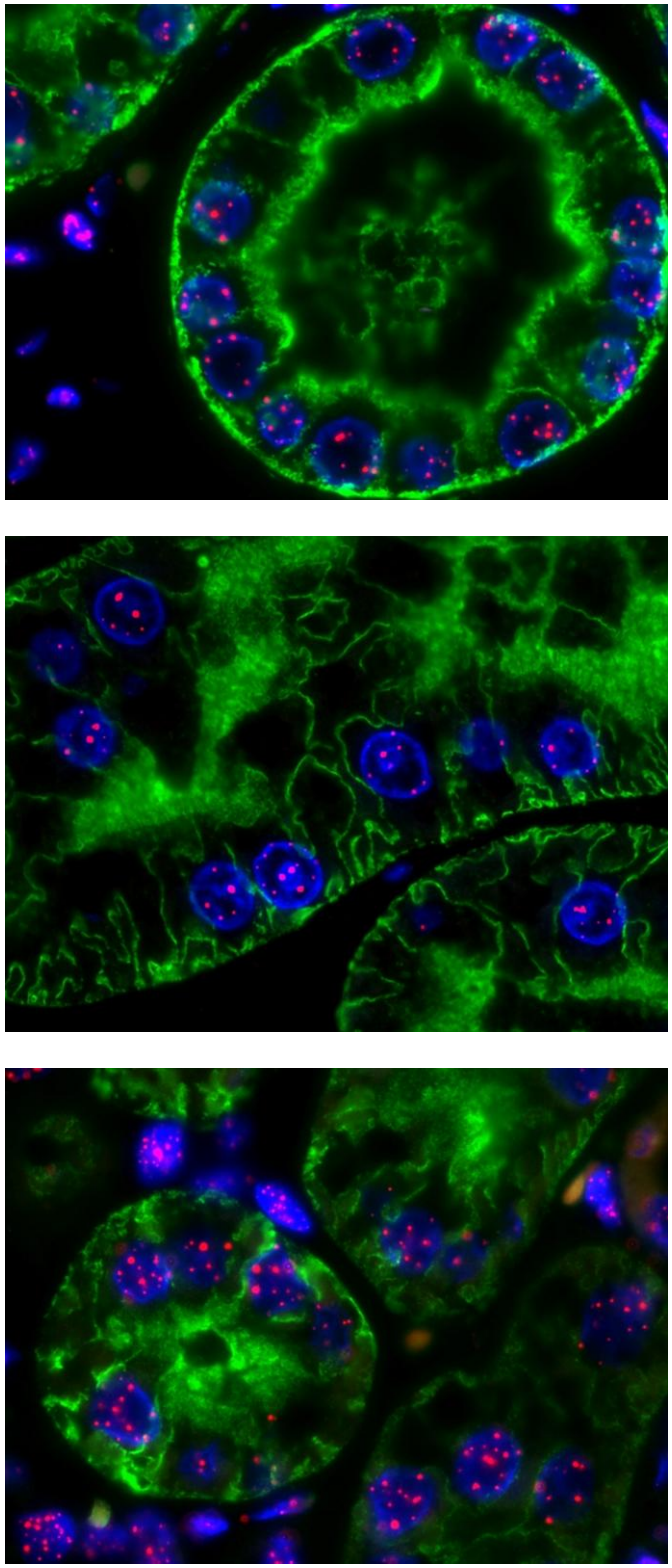


**Figure 3.3:** TELI-FISH analysis of aquaporin 1 positive proximal tubular cells in renal cortex of a CKD cat (top), normal cat (middle) and normal geriatric cat (bottom) (100x). Nuclei are blue and telomeric signals are pink dots within the blue nucleus.

*Average TFI analysis*

There was a statistically significant decrease in the average TFI of proximal tubular epithelial cells of CKD cats as compared to young normal or geriatric normal cats ($p = 0.0007$) (**Figure 3.4**). There was a statistically significant decrease in the average TFI in the distal tubular epithelial cells of CKD cats in comparison to young normal cats ($p = 0.004$) (**Figure 3.5**). No difference in average TFI was found between young normal and geriatric normal cats for either cell population.

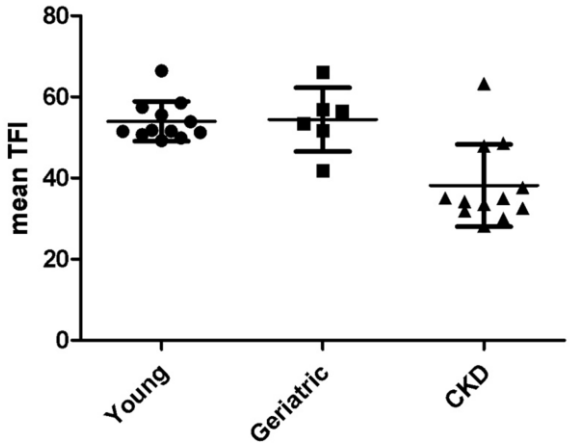



**Figure 3.4:** Average TFI of proximal tubular epithelial cells. Using a one way ANOVA with Dunn's post hoc analysis, a statistically significant decrease in the average TFI of proximal tubular epithelial cells of CKD cats in comparison to young normal and geriatric normal cats was demonstrated ($p = 0.0007$).

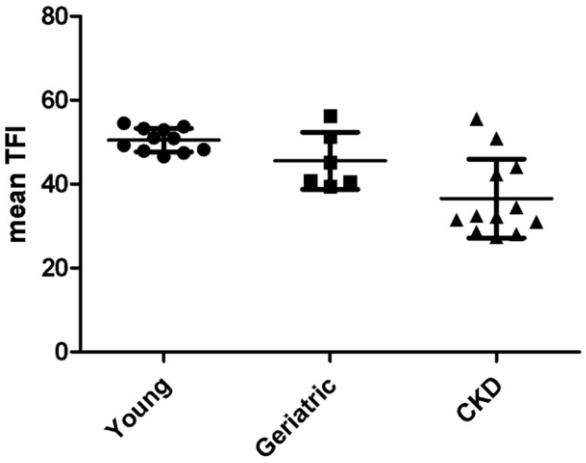



**Figure 3.5:** Average TFI of distal tubular epithelial cells. Using a one way ANOVA with Dunn's post hoc analysis, a statistically significant decrease in the average TFI of distal tubular epithelial cells of CKD cats in comparison to young normal and geriatric normal cats was demonstrated ($p = 0.004$).

*Frequency Histogram Analysis*

When histograms of individual kidney TFI were compared, there was a statistically significant decrease in the individual TFI of proximal tubular epithelial cells of CKD cats (mean: 36.9, CI: 36.4-37.4) in comparison to young normal (mean: 53.0, CI: 52.4-53.8) and geriatric normal cats (mean: 54.2, CI: 53.2-55) (**Figure 3.6**). There was also a statistically significant decrease in the individual TFI of distal tubular epithelial cells of CKD cats (mean: 35.8, CI: 35.3-36.2) in comparison to young normal (mean: 49.9, CI: 49.2-50.6) and geriatric normal cats (mean: 45.7, CI: 44.9-46.3) (**Figure 3.7)**. In addition, the distribution of the proximal tubule histogram was markedly different between CKD cats and normal cats. A bimodal distribution of TFI signals was apparent in the proximal tubule histograms from young normal and geriatric normal cats, but less so in CKD cats (**Figure 3.6**). This pattern was also present in the distal tubule histograms, but not as prominently as the former cell type. Further statistical analysis of this histogram pattern with a finite mixed model confirmed its bimodal nature and also allowed comparison of the individual distributions (**Figure 3.8**). Even when the histogram was broken down into two separate distributions (A = low end, B = high end), each individual distribution in the CKD cat group (mean A ± SE = 22.6 ± 0.24, mean B = 57.6 ± 0.78) was significantly different from those in young normal (mean A = 28.4 ± 0.44, mean B = 74.9 ± 1.1) and normal geriatric (mean A = 29.1 ± 0.57, mean B = 76.6 ± 1.4) cat groups when compared with a t test ($p = < 0.05$). Distribution B represents unusually large telomere signals that were visually observed in these sample groups. In CKD cats, it was estimated that only 40% of telomere signals were in distribution B, while 53% of telomere signals in young and geriatric cats were in distribution B.

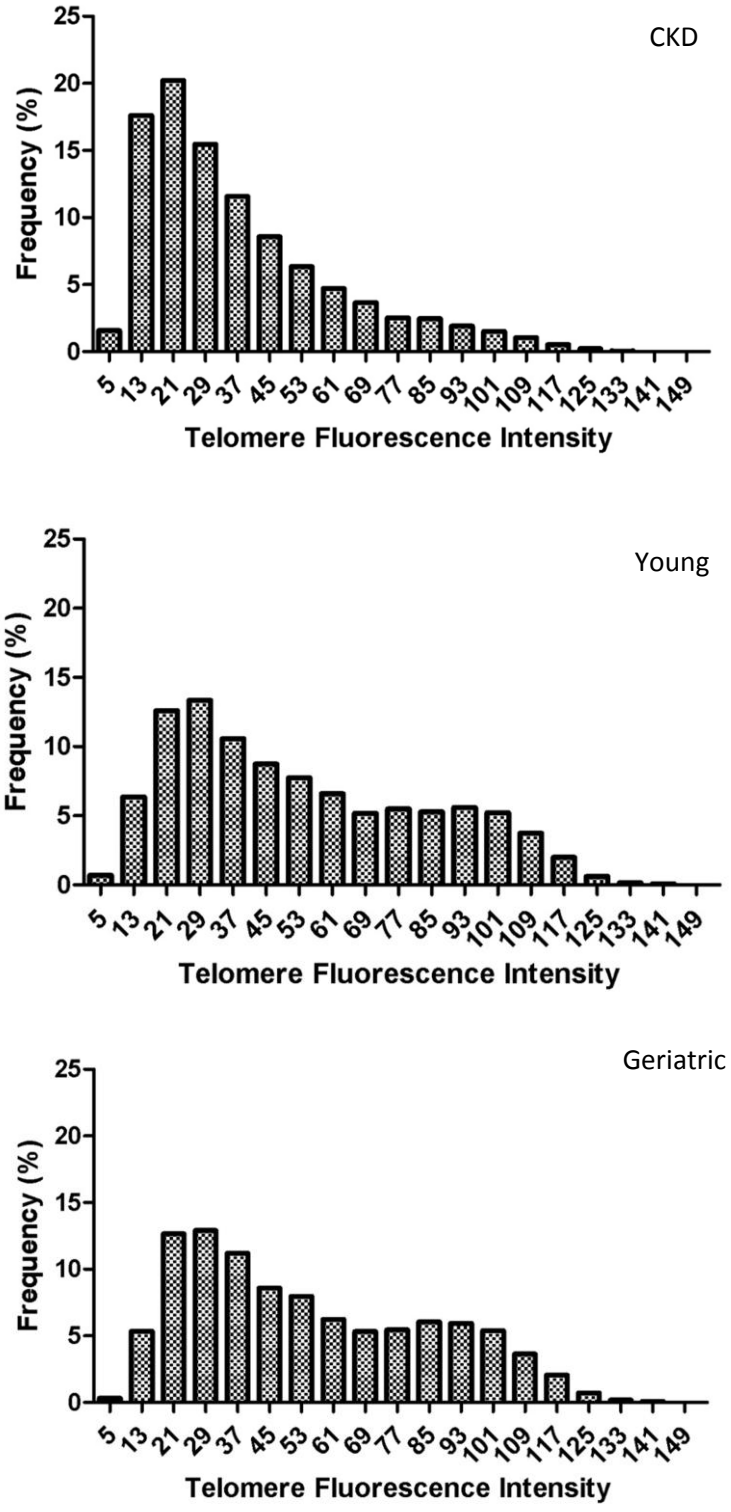



**Figure 3.6:** Proximal tubule individual TFI frequency histograms. A statistically significant decrease in the individual TFI of proximal tubular epithelial cells of CKD cats (mean: 36.9, CI: 36.4-37.4) is demonstrated in comparison to young normal (mean: 53.0, CI: 52.4-53.8) and geriatric normal cats (mean: 54.2, CI: 53.2-55).

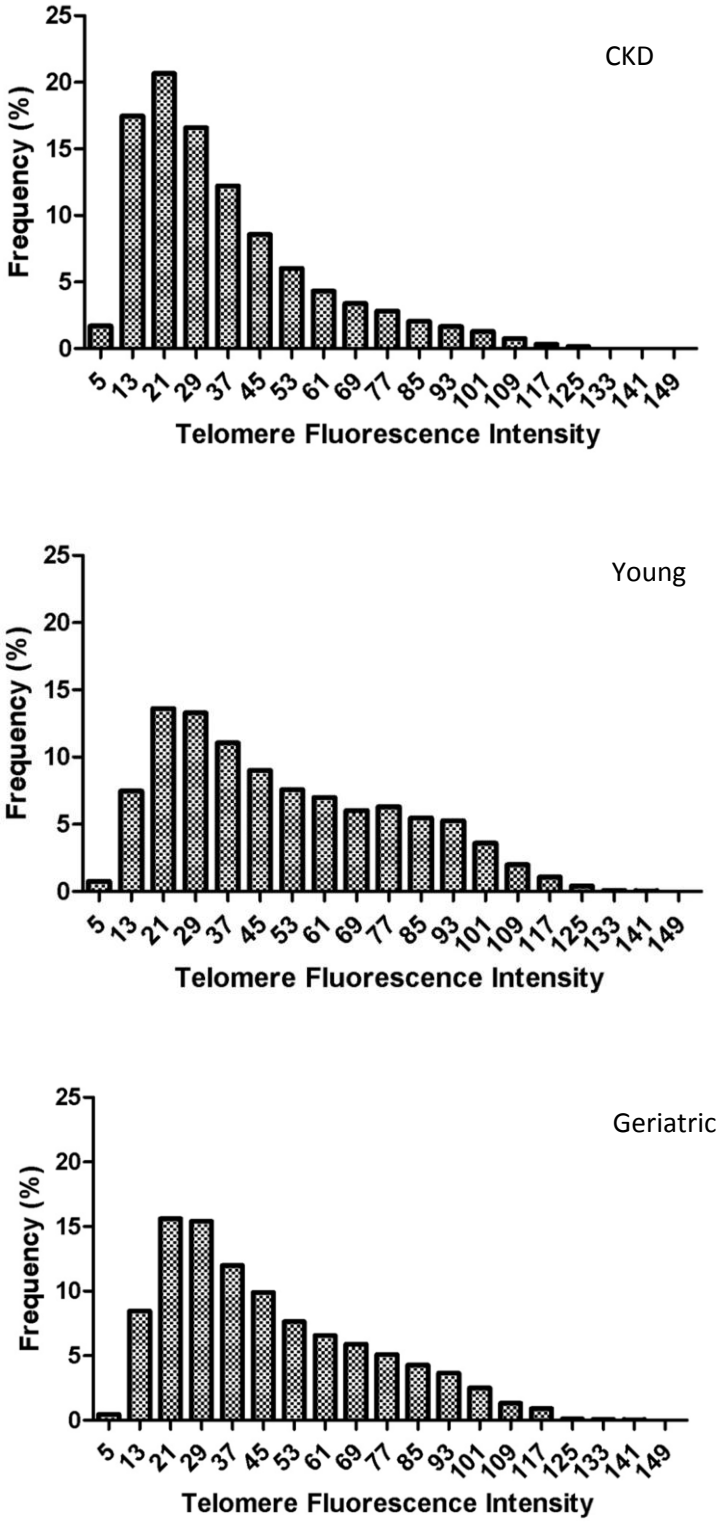
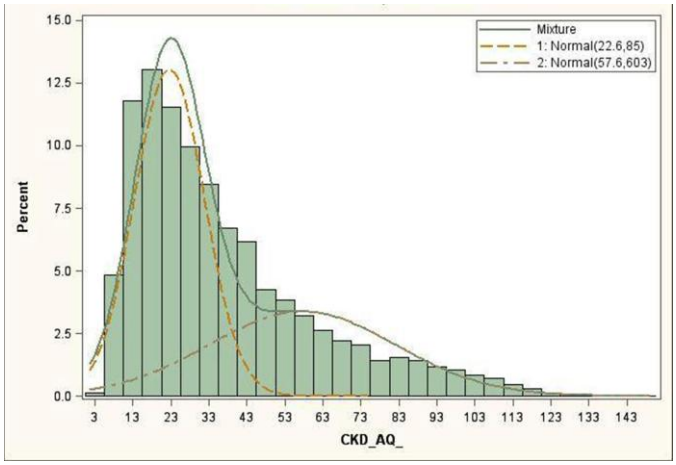
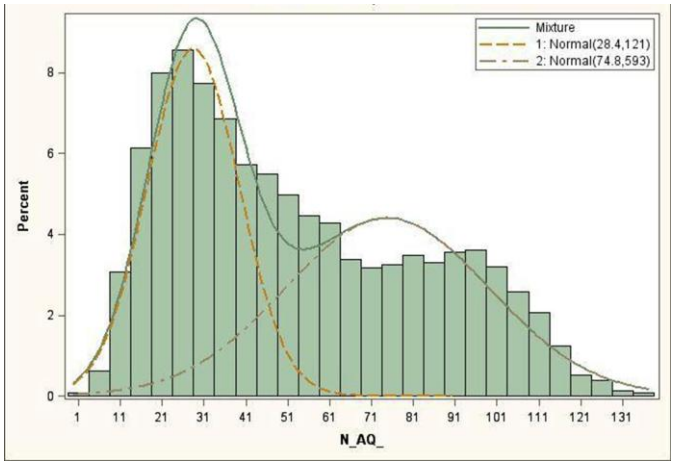



**Figure 3.7:** Distal tubule individual TFI frequency histograms. A statistically significant decrease in the individual TFI of distal tubular epithelial cells of CKD cats (mean: 36.9, CI: 36.4-37.4) is demonstrated in comparison to young normal (mean: 53.0, CI: 52.4-53.8) and geriatric normal cats (mean: 54.2, CI: 53.2-55).



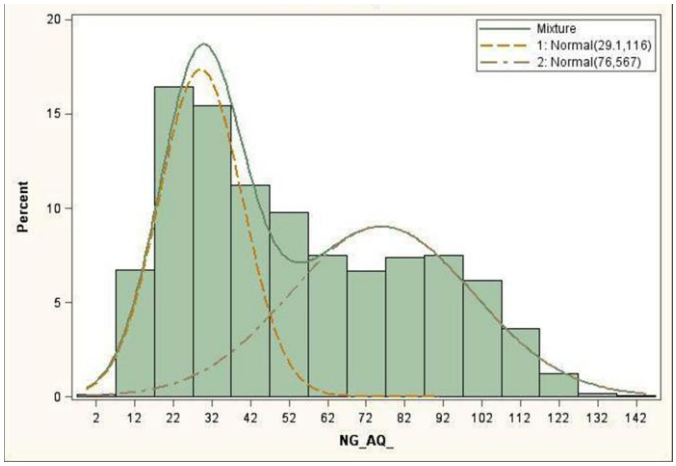




**Figure 3.8:** Finite mixed model analysis of bimodal distribution present in proximal tubule frequency histogram. Two distributions (A= low end, B = high end) were isolated from the histogram. Individual distributions in the CKD cat group (mean A ± SE = 22.6 ± 0.24, mean B = 57.6 ± 0.78) were significantly different from those in young normal (mean A = 28.4 ± 0.44, mean B = 74.9 ± 1.1) and normal geriatric (mean A = 29.1 ± 0.57, mean B = 76.6 ± 1.4) cat groups when compared with a t test ($p = < 0.05$). Distribution B represents unusually large telomere signals that were visually observed in these sample groups.

### *3.4.4 TEL-FISH Liver and Skin*

Telomere fluorescence in situ hybridization (TEL-FISH) was successfully performed on liver and skin sections. Hepatocyte nuclei were identified on DAPI images based on the morphologic appearance of the nucleus. Basal cell layer was identified in skin based on its undulating architecture. Adequate telomere signal for analysis was achieved using this technique (**Figure 3.9**). No difference in average TFI was found for liver or skin samples from any group (**Figures 3.10-11**). When histograms of individual liver TFI were compared, there was a statistically significant increase in the individual TFI of geriatric normal cats (mean: 36, CI: 36-37) in comparison to young normal (mean: 30, CI: 30-30) and CKD cats (mean: 29, CI: 29-30) (**Figure 3.12**) When histograms of individual skin TFI were compared there was no statistically significant difference in the individual TFI of CKD cats (mean: 32, CI: 32-33) in comparison to young normal (mean: 32, CI: 31-32) and geriatric normal cats (mean: 31, CI: 30-31) (**Figure 3.13**) A bimodal distribution was not appreciated in the frequency histograms for liver or skin.

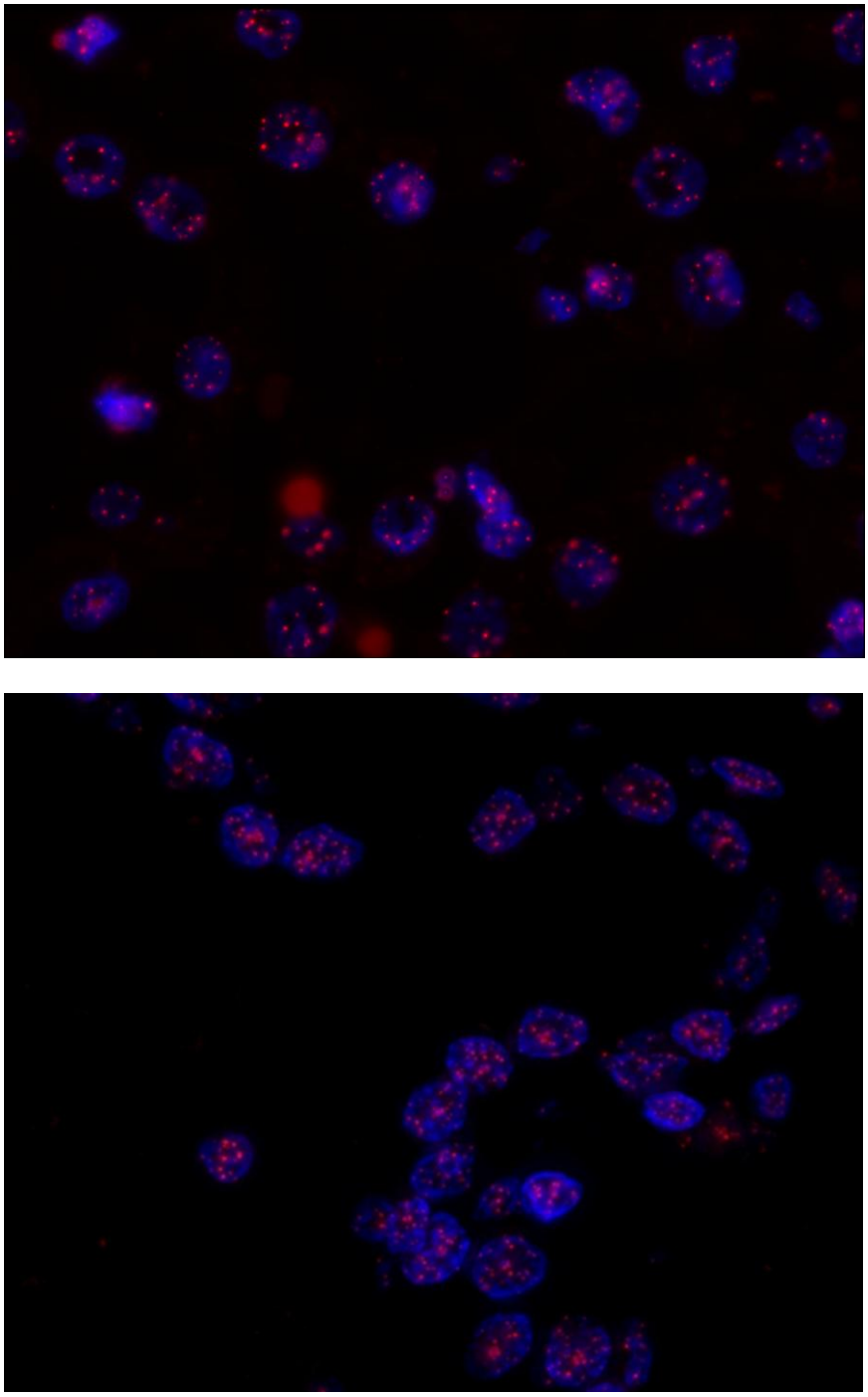


**Figure 3.9:** TEL-FISH analysis in the liver (top) and skin (bottom) of a young normal cat. Nuclei are blue and telomeric signals are pink dots within the blue nucleus.

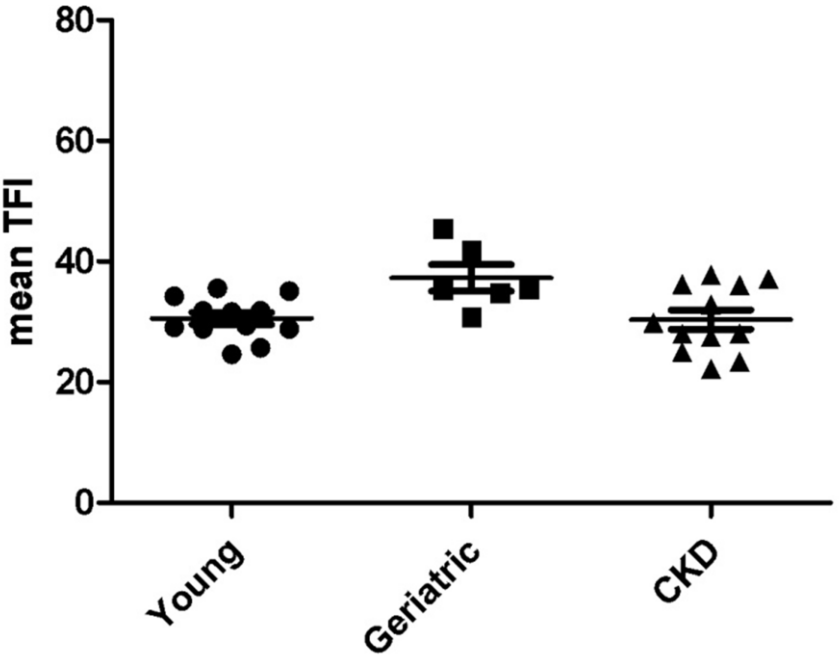



**Figure 3.10:** Average TFI of hepatocytes. Using a one way ANOVA with Dunn's post hoc analysis, no significant difference in the average TFI of hepatocytes between groups was demonstrated.

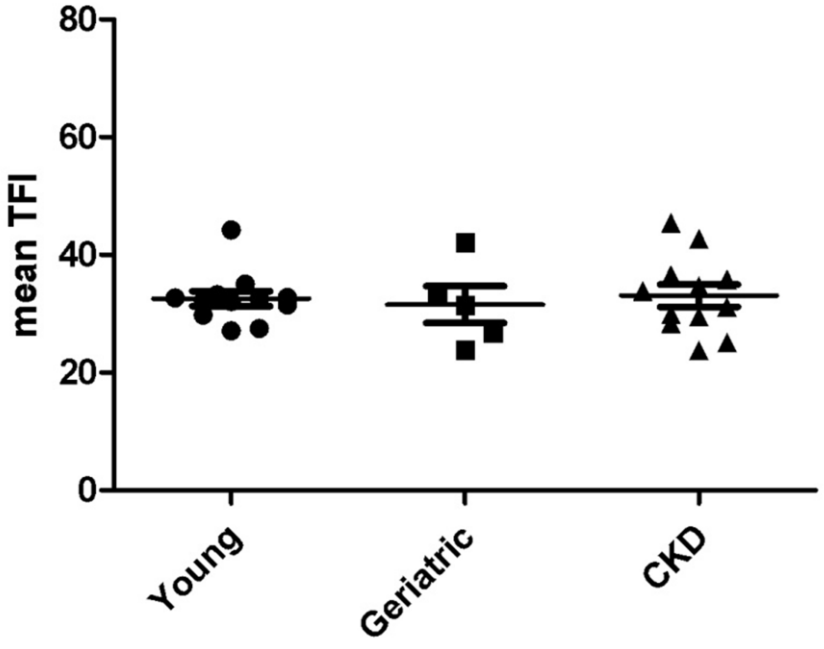



**Figure 3.11:** Average TFI of skin cells. Using a one way ANOVA with Dunn's post hoc analysis, no significant difference in the average TFI of skin cells between groups was demonstrated.

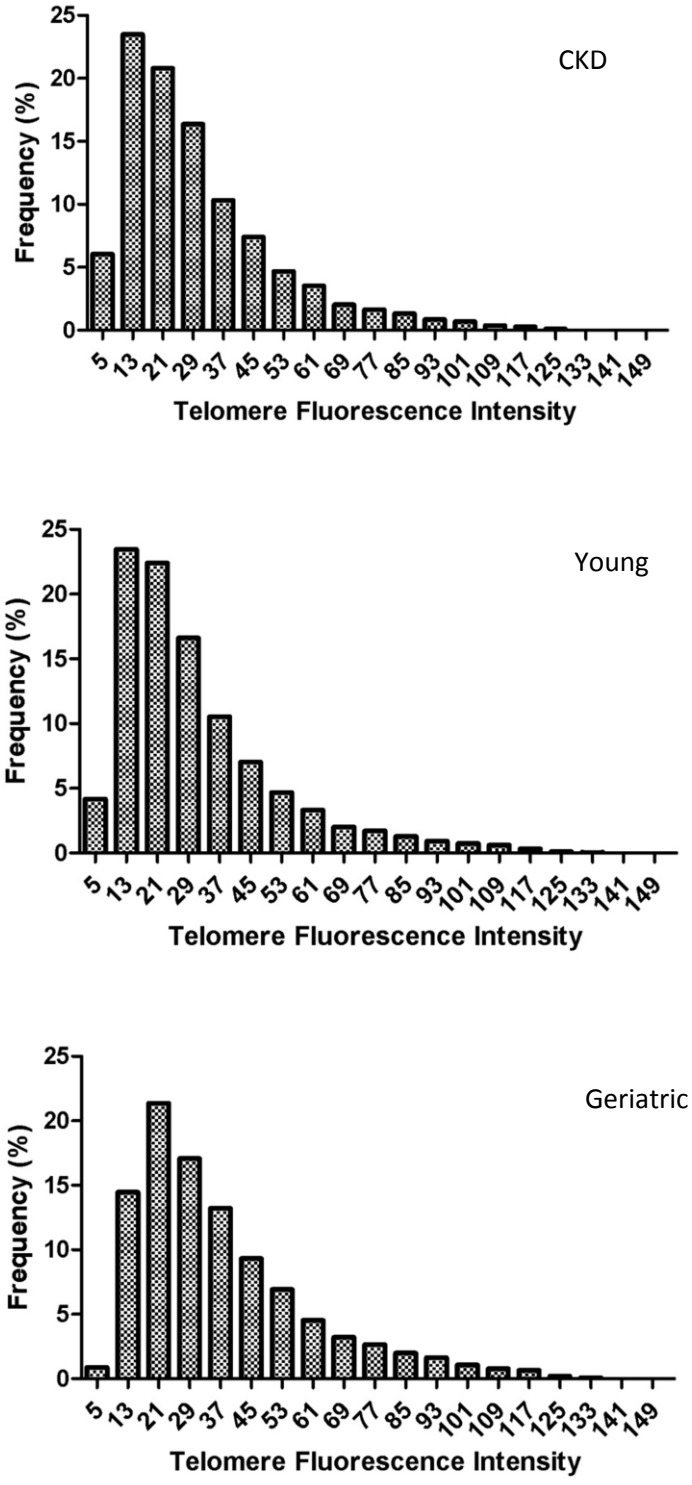



**Figure 3.12:** Hepatocyte individual TFI frequency histograms. There was a statistically significant increase in the individual TFI of geriatric normal cats (mean: 36, CI: 36-37) in comparison to young normal (mean: 30, CI: 30-30) and CKD cats (mean: 29, CI: 29-30) ($p = < 0.05$).

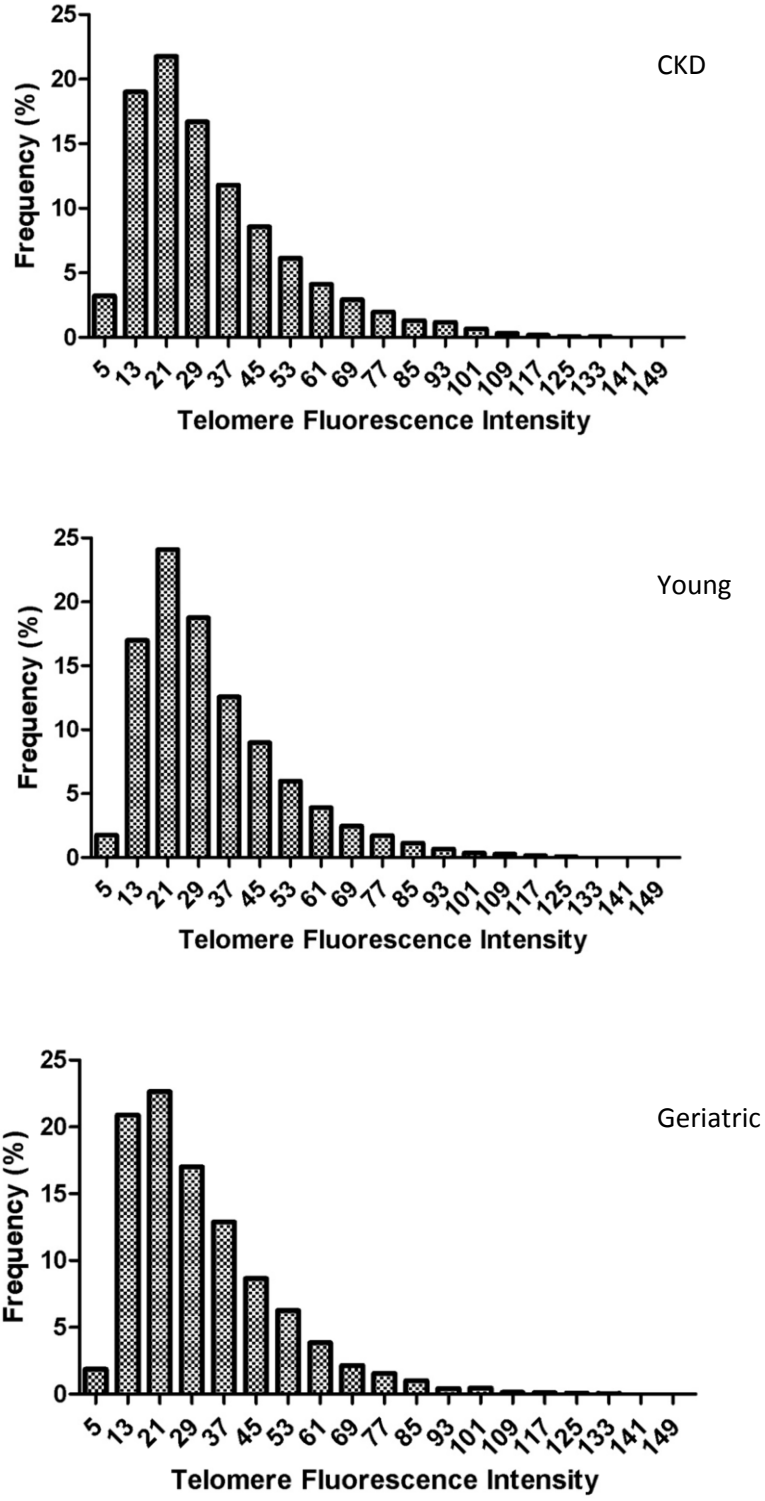



**Figure 3.13:** Skin individual TFI frequency histograms. No statistically significant difference is noted between cat groups.

### *3.5.5 β-galactosidase Analysis*

β-galactosidase analysis was performed on kidney and liver cryosections from 12 CKD cats, 12 young normal cats and 6 normal geriatric cats. Positive blue staining was seen prominently in tubular structures in the renal cortex of CKD cats. Some normal geriatric cats also exhibited staining of tubules. Representative pictures from each tissue and group are presented in **Figures 3.14-3.15**. When analyzed with a one way ANOVA with Dunn's post hoc analysis, there was a statistically significant increase in β-galactosidase staining in CKD kidney samples in comparison to young normal cats ($p = 0.0001$). CKD cats tended to have increased β-galactosidase staining in comparison to normal geriatric cats, but this did not reach statistical significance (**Figure 3.16**). Minimal β-galactosidase staining was seen in liver samples in comparison to kidney, however, CKD cats had a statistically significant increase in staining compared to other groups (**Figure 3.17**). Splenic tissue from CKD cats was also analyzed as a control to determine if myeloid cells would stain positive. Minimal β-galactosidase staining was present in the spleen (**Figure 3.18**). β-galactosidase staining and aquaporin 1 immunohistochemistry confirmed that proximal tubules were β-galactosidase positive (**Figure 3.19**).

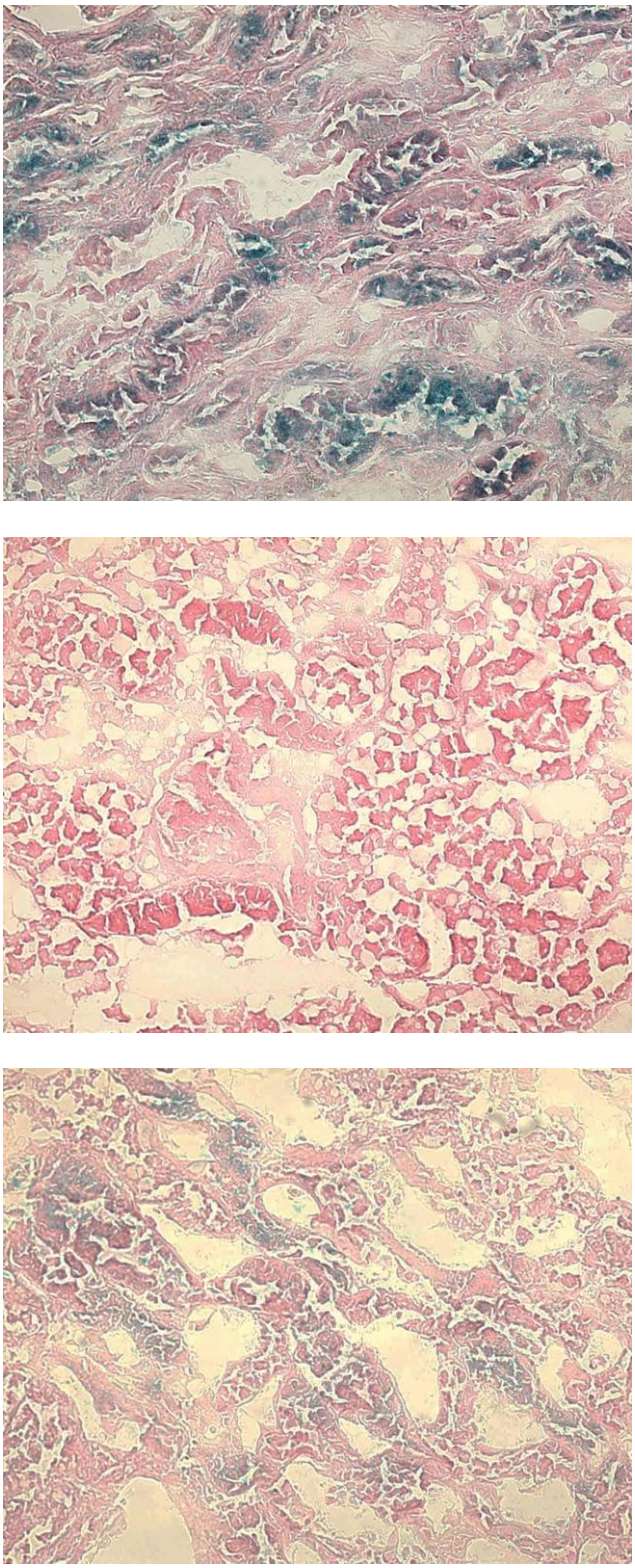


**Figure 3.14:** β-galactosidase staining in renal cortex of CKD cats (top), young normal cats (middle) and normal geriatric cats (bottom) (20x).

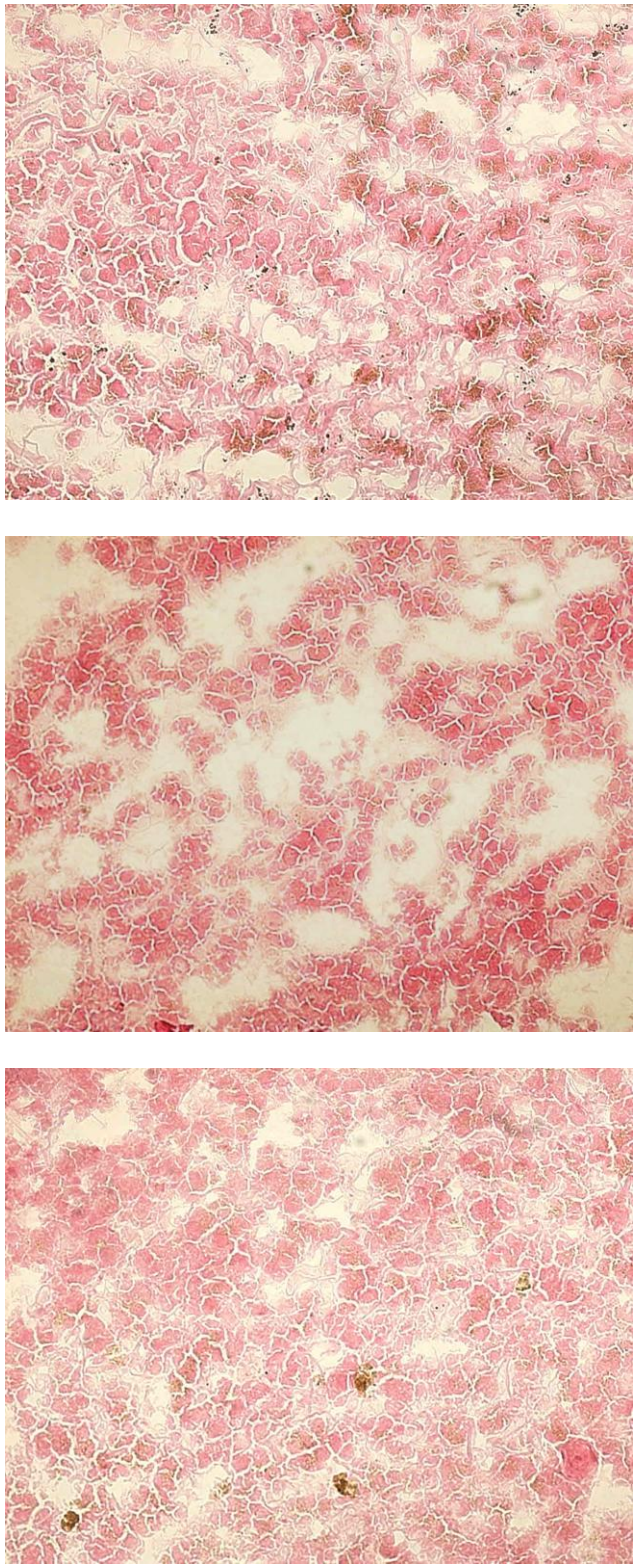


**Figure 3.15**: β-galactosidase staining in liver of CKD cats (top), young normal cats (middle) and normal geriatric cats (bottom) (20x).

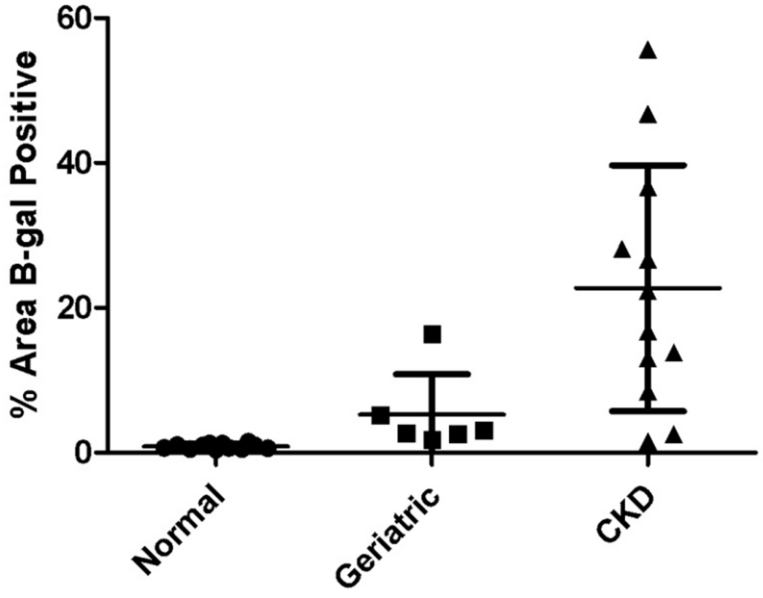



**Figure 3.16:** Image analysis of β-galactosidase staining in the kidney. Analysis of percent area positive was performed for four images from each sample, and an average calculated. There was a statistically significant increase in β-galactosidase staining in CKD kidney samples in comparison to young normal cats ($p = 0.0001$). CKD cats tended to have increased β-galactosidase staining in comparison to normal geriatric cats, but this did not reach statistical significance.

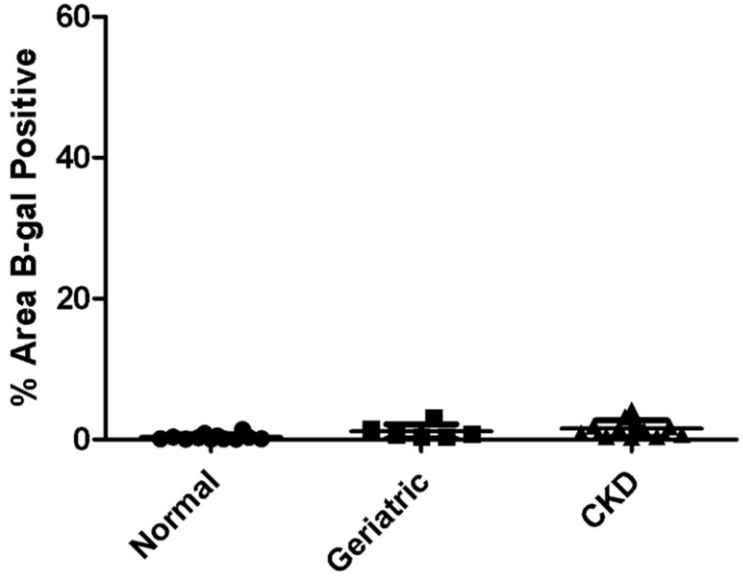



**Figure 3.17:** Image analysis of β-galactosidase staining in the liver. Analysis of percent area positive was performed for four images from each sample, and an average calculated. Minimal β-galactosidase staining was seen in liver samples compared to kidney, however, the CKD cats had a statistically significant increase in staining compared to other groups.

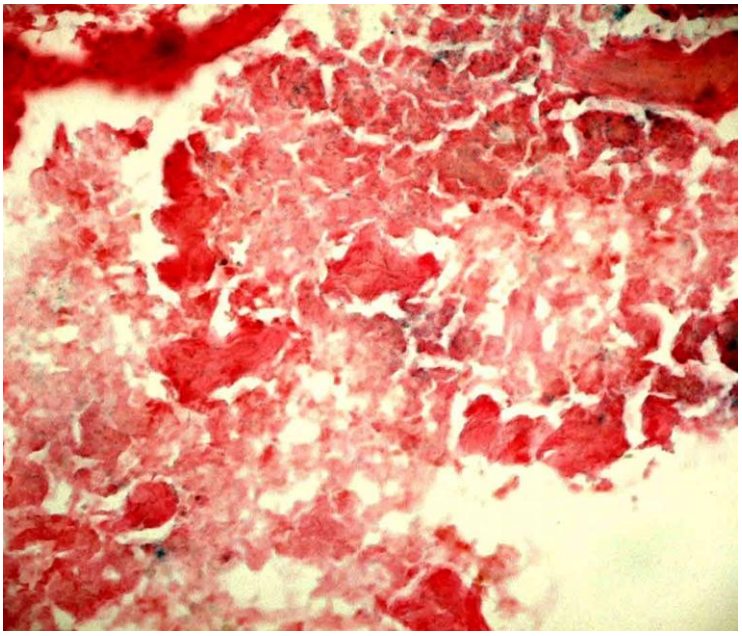


**Figure 3.18:** β-galactosidase staining in the spleen. Picture is representative of 6 samples analyzed as controls to determine if myeloid cells with also stain positive. Minimal staining was appreciated in the spleen.

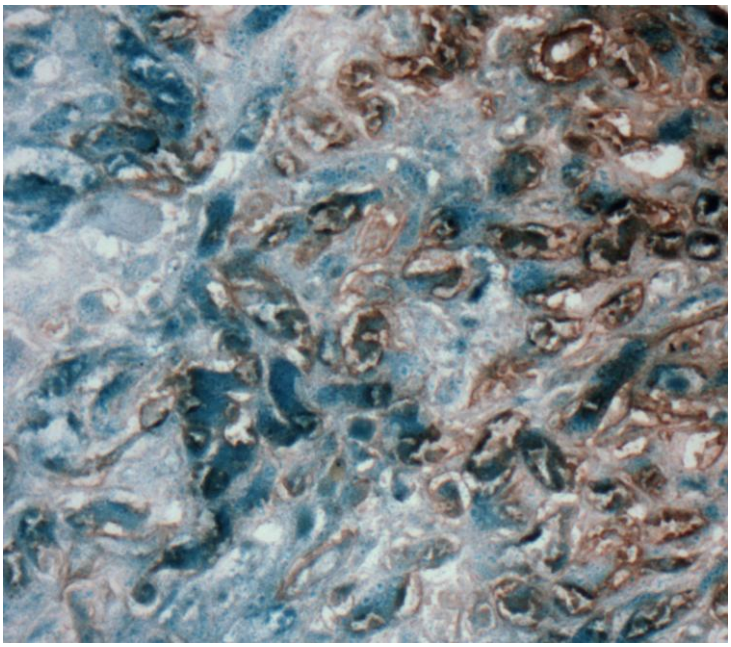


**Figure 3.19:** β-galactosidase staining combined with aquaporin 1 immunohistochemistry. Proximal tubular structures can be seen to stain both brown (aquaporin 1) and blue (β-galactosidase). (4x)

## 3.5 Discussion

In this study we demonstrated an association between shortened telomeres, increased cellular senescence and feline CKD. A novel approach, TELI-FISH, was adapted for use in cats to facilitate telomere length analysis of specific cellular compartments within the renal cortex. Analysis of telomere length in both proximal tubular and distal tubular cell populations demonstrated that they were significantly shorter (up to 2 times) in cats with CKD than either young normal cats or geriatric cats without evidence of CKD. Similar results were not seen in the liver, which was used as a control organ. Additionally, increased cellular senescence, as determined by β-galactosidase staining, was documented in cats with CKD in comparison to young normal cats. Increased cellular senescence was also found in the kidneys of normal geriatric cats, although not to the degree present in CKD cats. Minimal cellular senescence was seen in liver samples in comparison to kidney.

Telomere length in feline CKD has not previously been assessed, however as the renal environment in CKD is compromised by inflammation, intraglomerular hypertension, hypoxia and resulting oxidative stress, it is not surprising that the conclusions of the current study are consistent with previous literature in other species. In rats, oxidative stress, hypertension and renal ischemia have all been shown to be associated with shortened renal telomeres.[16-18] The results of β-galactosidase assays were also consistent with previous literature; increased renal senescence has been demonstrated in association with renal aging and disease in humans.[7,8,19]

A few studies in humans have examined the relationship between age and renal telomere attrition and concluded that telomeres shorten in an age-dependent manner, particularly in the renal cortex.[20,21] In cats, telomere length in relation to age has been investigated previously by TRF analysis of PBMC. A significant decrease in telomere length in PBMC was found with age.[22] In the current study, however, age effect alone did not appear to result in telomere shortening in the kidney or liver. Potentially this could be a result of the number of cats included in the study, as it was almost impossible to find geriatric cats that actually did not have CKD, therefore only 6 cats were included. Larger numbers and a greater range of ages would be ideal to more thoroughly investigate and establish the effect of age alone on renal telomere

length. Normal geriatric cats had slightly higher TFI in the liver than normal young cats. Some of this difference may be attributable to the amount of background present in the samples. It was subjectively noted that background was increased in young normal cats in comparison to other samples, which would affect telomere intensity signals by decreasing their measureable value.

The β-galactosidase assay employed to assess samples for senescence was problematic, as it can only be performed on cryosection samples and parenchymal architecture was not as well preserved in the cryosection samples as in the paraffin-embedded samples. It was difficult to determine exactly which cells were β-galactosidase positive as different cellular compartments were difficult to distinguish and individual tubular cells could not be identified. However, β-galactosidase positive tubular structures were identifiable and this was confirmed with the use of aquaporin 1 immunohistochemistry. It is unlikely that β-galactosidase staining of inflammatory infiltrates accounted for the increased staining seen in CKD cats as little β-galactosidase staining was seen in the spleen which contains large quantities of similar type cells. Ideally, future studies in feline CKD require optimization of a method for the measurement of senescence that could be performed on paraffin-embedded tissues so that a better assessment of which cells were senescent could be made. Unfortunately, similarly to our telomere length analysis, the small number of cats in the normal geriatric group likely limited and/or confounded our ability to completely assess the relationship between renal age and senescence. Although from the subjective appearance of the figure (**Figure 3.16**) there appears to be a difference in the degree of senescence between CKD cats and normal geriatric cats, this difference did not reach statistical significance. Additionally the large variance in the CKD cat results would have an effect on the ability to detect a difference between groups. Additional studies will be necessary to fully evaluate the relationship between renal age and senescence.

This project was unique in adapting TELI-FISH for cats, a relatively new technique for telomere length measurement that allows analysis of specific cellular compartments within the renal cortex. This approach conveys multiple advantages, including the ability to identify and analyze only nuclei of the cells of interest and the ability to visualize telomere location and appearance. Additionally, this technique can be used on paraffin embedded tissues which allows studies to be performed on previously archived

samples that have better architectural structure than frozen sections. Disadvantages of the technique that we experienced included susceptibility to background auto-fluorescence, which varied from organ to organ and from sample to sample. Additionally some variability in signal can be expected between samples, between sample runs and within the tissue itself (signal being brighter near the edge of the tissue). We attempted to control for these variables to the best of our ability by creating sets of samples for analytical runs that included one sample from each cat group and not obtaining images from the edges of tissues. Additionally all samples were analyzed on at least two occasions – once with TEL-FISH and once with TELI-FISH - and although the former data is not shown, the relative decrease in telomere signals in CKD cats remained constant regardless of the type of analysis or sample run.

The TELI-FISH technique also enabled visualization of the relative sizes and locations of telomere signals, and in this regard some intriguing results were noted. In young normal and normal geriatric cats, there was significant variability in the size of proximal tubule telomere signals, to the extent that there was a bimodal distribution appreciated on the individual TFI histograms. Proximal tubule nuclei tended to have 1-2 rather large telomere signals in additional to several smaller ones. This phenomenon was not appreciated in liver or skin samples from any of the groups. This potentially could be a technical phenomenon where individual telomeres were clumped together in the compressed deconvoluted picture. However, use of 3D stacked images circumvented this problem, and indeed when a 3D image of the cells was created, the larger telomere signals were still present. One possible explanation for this observation is that these signals represent telomeric aggregates. Telomeres do not normally form aggregates; each telomere has its own 3D space within the cell, and does not overlap with other telomeres even during interphase.[23] Telomeric aggregates have however been reported, particularly associated with cancer.[23] Aggregates are thought to lead to telomeric dysfunction as they result in anaphase bridging which can subsequently lead to deletions, amplifications, and triggering of apoptosis or senescence.[23] Although additional investigation of this phenomenon is clearly warranted, if variable telomere signals in feline renal cells represent telomeric aggregates, this could be a source of telomeric dysfunction that might predispose cats to developing CKD subsequent to renal injury and repair.

In summary, the results of this study are the first to demonstrate an association between telomere shortening, cellular senescence and feline CKD. Although these associations do not necessarily demonstrate a cause and effect relationship, they do reveal that telomere dysfunction may indeed contribute to predisposition for development of CKD. It is also possible that additional unknown factors predispose cats to renal insult and the resulting continued need for repair and replication in this organ leads to telomere shortening. Further studies will be necessary to explore the relationship between the two. Regardless, these studies suggest a novel intervention strategy and therapeutic target for treatment of CKD, in that telomerase activation may be useful in combating critical telomere shortening, thereby reducing cellular senescence and slowing kidney degeneration.

## 3.6 References


**1.** Perico N, Remuzzi G, Benigni A. Aging and the kidney. *Curr Opin Nephrol Hypertens.* May 2011;20(3):312-317.

**2.** Schmitt R, Coca S, Kanbay M, Tinetti ME, Cantley LG, Parikh CR. Recovery of kidney function after acute kidney injury in the elderly: a systematic review and meta-analysis. *Am J Kidney Dis.* Aug 2008;52(2):262-271.

**3.** Ferlicot S, Durrbach A, Ba N, Desvaux D, Bedossa P, Paradis V. The role of replicative senescence in chronic allograft nephropathy. *Hum Pathol.* Sep 2003;34(9):924-928.

**4.** de Fijter JW, Mallat MJ, Doxiadis, II, et al. Increased immunogenicity and cause of graft loss of old donor kidneys. *J Am Soc Nephrol.* Jul 2001;12(7):1538-1546.

**5.** Jiang H, Ju Z, Rudolph KL. Telomere shortening and aging. *Z Gerontol Geriatr.* Oct 2007;40(5):314-324.

**6.** Houben JM, Moonen HJ, van Schooten FJ, Hageman GJ. Telomere length assessment: biomarker of chronic oxidative stress? *Free Radic Biol Med.* Feb 1 2008;44(3):235-246.

**7.** Verzola D, Gandolfo MT, Gaetani G, et al. Accelerated senescence in the kidneys of patients with type 2 diabetic nephropathy. *Am J Physiol Renal Physiol.* Nov 2008;295(5):F1563-1573.

**8.** Melk A, Schmidt BM, Takeuchi O, Sawitzki B, Rayner DC, Halloran PF. Expression of p16INK4a and other cell cycle regulator and senescence associated genes in aging human kidney. *Kidney Int.* Feb 2004;65(2):510-520.

**9.** Naesens M. Replicative senescence in kidney aging, renal disease, and renal transplantation. *Discov Med.* Jan 2011;11(56):65-75.

**10.** Meeker AK, Gage WR, Hicks JL, et al. Telomere length assessment in human archival tissues: combined telomere fluorescence in situ hybridization and immunostaining. *Am J Pathol.* Apr 2002;160(4):1259-1268.

**11.** DiBartola SP, Rutgers HC, Zack PM, Tarr MJ. Clinicopathologic findings associated with chronic renal disease in cats: 74 cases (1973-1984). *J Am Vet Med Assoc.* May 1 1987;190(9):1196-1202.

**12.** Keegan RF, Webb CB. Oxidative stress and neutrophil function in cats with chronic renal failure. *J Vet Intern Med.* May-Jun 2010;24(3):514-519.

**13.** Brandt LE, Bohn AA, Charles JB, Ehrhart EJ. Localization of Canine, Feline, and Mouse Renal Membrane Proteins. *Vet Pathol.* Jun 28 2011.

**14.** Reyes JL, Lamas M, Martin D, et al. The renal segmental distribution of claudins changes with development. *Kidney Int.* Aug 2002;62(2):476-487.

**15.** Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A.* Sep 26 1995;92(20):9363-9367.

**16.** Tarry-Adkins JL, Ozanne SE, Norden A, Cherif H, Hales CN. Lower antioxidant capacity and elevated p53 and p21 may be a link between gender disparity in renal telomere shortening, albuminuria, and longevity. *Am J Physiol Renal Physiol.* Feb 2006;290(2):F509-516.

**17.** Joosten SA, van Ham V, Nolan CE, et al. Telomere shortening and cellular senescence in a model of chronic renal allograft rejection. *Am J Pathol.* Apr 2003;162(4):1305-1312.

**18.** Hamet P, Thorin-Trescases N, Moreau P, et al. Workshop: excess growth and apoptosis: is hypertension a case of accelerated aging of cardiovascular cells? *Hypertension.* Feb 2001;37(2 Part 2):760-766.

**19.** Melk A, Schmidt BM, Vongwiwatana A, Rayner DC, Halloran PF. Increased expression of senescence-associated cell cycle inhibitor p16INK4a in deteriorating renal transplants and diseased native kidney. *Am J Transplant.* Jun 2005;5(6):1375-1382.

**20.** Melk A, Ramassar V, Helms LM, et al. Telomere shortening in kidneys with age. *J Am Soc Nephrol.* Mar 2000;11(3):444-453.

**21.** Tchakmakjian L, Gardner JP, Wilson PD, et al. Age-dependent telomere attrition as a potential indicator of racial differences in renal growth patterns. *Nephron Exp Nephrol*. 2004;98(3):e82-88.
**22.** McKevitt TP, Nasir L, Wallis CV, Argyle DJ. A cohort study of telomere and telomerase biology in cats. *Am J Vet Res*. Dec 2003;64(12):1496-1499.
**23.** Mai S, Garini Y. The significance of telomeric aggregates in the interphase nuclei of tumor cells. *J Cell Biochem.* Apr 1 2006;97(5):904-915.

# CHAPTER 4: MIRTAZAPINE AS AN APPETITE STIMULANT AND ANTI-NAUSEA THERAPY FOR CATS WITH CHRONIC KIDNEY DISEASE

I would like to acknowledge the contribution of Dr. Dan Gustafson and members of the Pharmacology Core for developing a protocol for analysis of serum mirtazapine levels and pharmacokinetic analysis.

## 4.1 Abstract

**Background:** Cats with CKD often experience inappetence, and may benefit from administration of mirtazapine, an appetite stimulant. However, the pharmacokinetic and pharmacodynamic properties of mirtazapine in CKD cats are unknown.

**Aims:** The aims of these studies were to a) elucidate the pharmacokinetics of mirtazapine in young normal, geriatric and CKD cats; b) determine the appetite-stimulating effects and adverse effects of mirtazapine in normal cats; c) evaluate the efficacy of mirtazapine for increasing body weight and appetite in CKD cats.

**Methods:** A pharmacokinetic study was conducted in 10 normal cats, 6 CKD cats and 6 age-matched controls (AMC). 3.75 mg (High dose: HD) or 1.88 mg (Low Dose: LD) of mirtazapine was administered to young normal cats and LD only was administered to AMC and CKD cats. Mirtazapine concentrations were measured by liquid chromatography coupled to tandem mass spectrometry. Non-compartmental pharmacokinetic modeling was performed. Pharmacodynamic effects were studied in 14 healthy cats administered placebo, LD or HD mirtazapine in a crossover, blinded trial. Lastly, a placebo-controlled blinded crossover clinical trial was conducted in 11 cats with CKD.

**Results:** Mean half-life of mirtazapine was 15.3 +/- 4.7 hours (HD) and 10.3 +/- 2.3 hours (LD) in normal cats. There was a statistically significant difference between the elimination half-life, clearance,

AUC/Dose, and $AUC_{\infty}$/Dose of the high dose and low dose groups when compared with Mann-Whitney statistics. Mean half-life +/- SD was 15.2 +/- 4.2 hours in CKD cats and 12.1 +/- 1.1 hours in AMC cats. There was a statistically significant difference in AUC and clearance between normal geriatric cats and CKD cats. The half-life of mirtazapine was compatible with daily dosing in normal cats and LD mirtazapine was administered daily for 6 days with no drug accumulation detected. In the pharmacodynamic study normal cats that received mirtazapine ingested significantly more food than cats that received placebo. No difference in food intake was seen between HD and LD, but significantly more behavior changes were seen with the HD. Limited sampling during the pharmacodynamic study revealed AUC ranges comparable with the pharmacokinetic study. In the clinical trial, CKD cats receiving mirtazapine had a statistically significant increase in weight, appetite and activity and a decrease in vomiting compared to placebo.

**Conclusions:** Mirtazapine is an effective appetite stimulant in cats. Lower doses results in fewer side effects with similar clinical efficacy. CKD delays the clearance of mirtazapine. Low dose mirtazapine had a half-life compatible with a 48-hour dosing interval in CKD cats and resulted in significantly increased appetite and weight.

## 4.2 Introduction

Clinical signs of feline CKD include polyuria, polydipsia, decreased appetite, weight loss and vomiting. Currently no treatment other than renal transplantation has been shown to reverse or halt declining renal function for any significant period of time. Medical management therefore is the mainstay of treatment and can help patients cope with metabolic complications of the disease and improve quality of life.[1,2] Metabolic consequences and imbalances such as azotemia, hypertension, proteinuria, hypokalemia, hyperphosphatemia, anemia and dehydration progressively worsen with disease stage and may affect appetite. Management of these complications is a core part of medical therapy for these patients.[2] Nutrition is important for long term prognosis and efforts to directly target nausea and appetite, in addition to other medical therapies will undoubtedly benefit patients.

In the chronically ill patient, and specifically in CKD, poor body condition has been correlated with decreased survival.[3-5] Nutritional support is a key component of patient management in CKD. Several recent studies have documented the therapeutic value of specially formulated diets in the management of CKD.[6-8] These diets typically contain restricted amounts of high quality protein, adequate non-protein calories, and are restricted in phosphorus.[8] The failure of the patient to eat the diet negates the benefit of dietary management, and therefore a key therapeutic target for these patients is the maintenance of appetite and food intake. Current strategies to enhance appetite include the use of $H_2$-receptor antagonists or proton pump inhibitors to manage uremic gastritis, and cyproheptadine as an appetite stimulant.[9] Feeding tubes may also be used, but are not an acceptable option for many pet owners.

Mirtazapine, a pre-synaptic $\alpha$2-adrenergic receptor antagonist, was originally introduced to human medicine as an antidepressant; however it has recently attracted interest in veterinary medicine due to several desirable effects, namely its significant anti-nausea, anti-emetic, and appetite stimulating properties. These effects appear to be a result of antagonism of the 5-$HT_3$ receptor, which is an important receptor in the physiology of emesis. Noradrenergic effects appear to be responsible for the antidepressant properties.[10]

An initial uncontrolled clinical trial[11] and numerous anecdotal reports have encouraged continued clinical use of mirtazapine in veterinary medicine. Doses for cats and dogs have been extrapolated from human doses. A dose of 1.88 or 3.75 mg (1/8 or 1/4 of a 15 mg tablet) every three days has been recommended for cats,[11] however no pharmacological studies have been reported to date in support of these extrapolations. Anecdotal observations in our clinic noted that in most cases the effect of the drug appears to have dissipated by the second day after administration, implying a shorter dosing interval may be more appropriate.

Human pharmacokinetic data have demonstrated that a number of factors affect the metabolism of mirtazapine. Differences in metabolism were found between sexes and ages, and for patients suffering from hepatic or renal impairment.[10] In order to provide accurate dosing recommendations it would therefore be prudent to investigate the pharmacokinetics of mirtazapine in veterinary patients. This is

particularly important in feline patients as the drug may be metabolized more slowly due to the decreased availability of some metabolic pathways in this species. For example, cats lack glucuronyl transferase which is necessary for glucuronide conjugation.[12] As mirtazapine is metabolized initially by demethylation and oxidation, followed by conjugation to glucuronic acid, some differences in drug metabolism and elimination for felines might be expected.[10] Therefore this project was designed to assess the pharmacokinetics, efficacy, and safety of commonly prescribed doses of mirtazapine to provide information to guide the use of mirtazapine as an appetite stimulant in cats, particularly for those suffering from CKD. Specifically we sought to determine the pharmacokinetics of mirtazapine in young normal cats, geriatric normal cats and cats with CKD (Section 4.3). A pharmacodynamic study was subsequently performed in normal cats to assess efficacy and side effects of the doses investigated in the pharmacokinetic study (Section 4.4). Finally a placebo controlled blinded crossover clinical trial was performed in CKD cats to document efficacy of mirtazapine (Section 4.5).

## 4.3 Pharmacokinetics of Mirtazapine in Young Normal Cats, Geriatric Normal Cats and Cats with CKD

### 4.3.1 Materials and Methods

#### *4.3.1a.Cats.*

*Normal Cats*

Seven purpose-bred and 3 privately owned young adult healthy cats were enrolled in the pharmacokinetic study of normal animals after undergoing a physical examination, complete blood count, serum biochemistry profile and urinalysis to rule out systemic disease. Four cats from the seven purpose-bred cats that participated in the pharmacokinetic study were utilized for the daily administration study. All portions of the study were approved by the Institutional Animal Care and Use Committee at Colorado State University, and all owners signed consent forms prior to participation.

*CKD Cats and Age-Matched Control Cats*

Six client-owned stable CKD cats, two each from International Renal Interest Society (IRIS) stages II, III and IV, and 6 age-matched (within 6 months) healthy geriatric control cats (AMC) were enrolled through stratified convenience sampling. Cats were considered to have stable CKD if serum creatinine had not changed by more than 10% on at least two measurements in the previous 60 days. Diagnostic tests required before enrollment included a minimum database consisting of serum biochemistry profile, complete blood count, urinalysis, urine culture, blood pressure, and serum total thyroxine measurement. Healthy control cats were defined as those with no clinical abnormalities, normal minimum database including a creatinine < 1.8 mg/dL and a urine specific gravity greater than 1.035. Exclusion criteria included other systemic illnesses, complications of CKD such as hypertension, pyelonephritis or ureteral obstruction, or decompensation of CKD requiring hospitalization and intravenous fluid therapy. All portions of the study were approved by the Institutional Animal Care and Use Committee at Colorado State University, and all owners signed consent forms prior to participation.

### *4.3.1b. Drug Preparation.*

Commercially available generic 15 mg mirtazapine (Aurobindo Pharma USA, Inc., Dayton, NJ) tablets were compounded by the pharmacy at the Colorado State University Veterinary Medical Center according to Professional Compounding Centers of America® protocol. The method used is guaranteed to produce accurate compounding within 10% of the target dose. Analysis of random compounded capsules for mirtazapine content using liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) showed accuracies of 94.5 ± 4.6% to the intended content and a stability of at least 6 months as formulated. The capsules were compounded within one month of utilization and stored at room temperature.

### *4.3.1c Pharmacokinetic Study Design.*

For the normal cat pharmacokinetic study, ten cats were randomly assigned to either the high dose (HD) group that received 3.75 mg, or to the low dose (LD) group that received 1.88 mg of mirtazapine. Capsules were administered orally, followed by 3 mL of water given with a syringe. The cats were fasted for 12 hours before beginning the study. A jugular catheter was placed under ketamine (20mg per cat IV) and butorphanol (0.1mg/kg IV) sedation three hours prior to mirtazapine administration to allow for ease of sample collection. Blood samples (1.5 mL) were obtained prior to, and at 0.25, 0.5, 1, 4, 8, 24, 48, and 72 hours after mirtazapine administration. Samples were centrifuged within 10 minutes of collection and serum was harvested and stored at –80°C until analysis.

For the CKD and normal geriatric pharmacokinetic study the cats were fasted for 12 hours before beginning the study. A jugular catheter was placed under ketamine (20mg per cat IV) and butorphanol (0.1mg/kg IV) sedation three hours prior to mirtazapine administration, to allow for ease of sample collection. A capsule containing 1.88 mg of mirtazapine was administered orally once, followed by 3 mL of water administered by syringe. Blood samples (1.0 mL) were obtained prior to, and at 0.5, 1, 1.5, 2, 4,

8, 24, and 48 hours after mirtazapine administration. Samples were centrifuged within 10 minutes of collection and serum was harvested and stored at –80°C until analysis.

#### *4.3.1d. Daily Administration Study.*

Based on the information gathered from the pharmacokinetic and pharmacodynamic studies, LD mirtazapine was administered daily to determine if drug accumulation would occur. Four cats (2 female, 2 male) received LD mirtazapine orally once daily for 6 days. On days 3 and 6, blood samples were drawn immediately before, and 2 hours after, capsule administration and processed as previously described.

#### *4.3.1e. Mirtazapine Analysis.*

Mirtazapine was measured using liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) using a method modified from one previously published.[13] The analysis was carried out in the Pharmacology Core at the Colorado State University Veterinary Medical Center. An LC/MS/MS based assay was developed and validated for the analysis of mirtazapine in cat serum. For sample preparation, a 200 µL aliquot of serum was transferred to a 1.5 mL polypropylene microcentrifuge tube and 10 ng of trazodone (10 µL of 1 µg/mL) was added as an internal standard. Proteins were then precipitated by the addition of 500 µL of acetonitrile and vortexing for 10 minutes. Samples were centrifuged at 13,000 RPM for 10 minutes and the resulting supernatant collected. Standards and quality assurance/quality control (QA/QC) samples were constructed by spiking 200 µL of blank cat serum with known amounts of mirtazapine and processing as described above.

Positive ion electrospray ionization mass spectra were obtained with a 3200 Q-TRAP triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with a turbo ionspray source interfaced to a 1200 Series Binary Pump SL liquid chromatography system (Agilent Technologies Inc, Santa Clara, CA) and HTC-PAL autosampler (Leap Technologies, Carrboro, NC). Chromatography was performed on a SunFire C18, 2.5 µm, 4.6 x 50 mm column (Waters Corporation, Milford, MA) with a

liquid chromatography gradient employing 10 mM ammonium acetate (mobile phase A) and acetonitrile (mobile phase B). Initial chromatographic conditions were 60:40 (mobile phase A: mobile phase B) with increasing B from 40% to 95% from 0 to 2 minutes, holding at 95% B from 2 to 2.75 minutes, and re-establishing initial conditions from 2.75 to 4 minutes. The flow rate was 1 mL/minute, injection volume was 4-20 µL, and the total analysis time was 4 minutes.

Assay performance for each batch was assessed utilizing at least 10% QA/QC samples dispersed amongst unknown samples at low (1 ng/ml), mid (10 ng/ml) and high (100 ng/ml) ranges of the standard curve (0.5-500 ng/ml) with batches failing if >25% of the QA/QC samples were outside of the accepted level of 85% accuracy. Accuracy of QA/QC samples amongst the batches analyzed for this study ranged from 94.5 ± 4.6% to 92.2 ±6.8%. The lower limit of quantitation (LLOQ) for this assay was based on the level of detection with >85% accuracy and a CV (%) <15% and was determined to be 0.5 ng/ml. Assay performance was linear to >500 ng/ml, but 500 ng/ml was used as the upper limit of the assay as utilized, due to no unknown sample measurements exceeding this value.

The mass spectrometer settings were: turbo ionspray temperature, 550°C; ion-spray voltage, 1500 V; declustering potential, 48 V; entrance potential, 4.5 V; collision energy, 37 V; collision cell exit potential, 2 V; collision cell entrance potential, 11 V; collision gas, $N_2$, medium; curtain gas, $N_2$, 35 units; nebulizer gas, $N_2$, 40 units; and auxiliary gas, $N_2$, 20 units. Samples were quantified by the internal standard reference method in the multiple reaction monitoring mode by monitoring the transition *m/z* 266 $\rightarrow$ 195 for the analyte mirtazapine and *m/z* 372 $\rightarrow$ 176 for the internal standard trazodone.

#### *4.3.1f. 8-Hydroxymirtazapine and 8-Hydroxymirtazapine Glucuronide Analysis.*

For the analysis of 8-hydroxymirtazapine and 8-hydroxymirtazapine glucuronide, aliquots of cat serum samples were treated with either β-glucuronidase or saccharic acid. The addition of saccharic acid to samples was for inhibition of endogenous glucuronidase activity. To each 100 µL aliquot of cat serum was added 1 ng of trazodone (10 µL of 100 ng/mL) followed by either 2500 units (50 µl of 50 units/µL) of β-glucuronidase or 50 µL of 300 µM saccharic acid. Samples were vortexed briefly to mix and

incubated at 37°C for 1 hour. Samples were then prepared by acetonitrile protein precipitation as described for mirtazapine.

Chromatographic conditions used were identical to those used for mirtazapine, and the mass spectrometer settings used were: turbo ionspray temperature, 350°C; ion-spray voltage, 1500 V; declustering potential, 48 V; entrance potential, 7 V; collision energy, 37 V; collision cell exit potential, 2 V; collision cell entrance potential, 11 V; collision gas, $N_2$, low; curtain gas, $N_2$, 35 units; nebulizer gas, $N_2$, 40 units; and auxiliary gas, $N_2$, 20 units. 8-hydroxymirtazapine was monitored as a transition *m/z* 282 $\rightarrow$ 211. The relative amount of 8-hydroxymirtazapine and 8-hydroxymirtazapine glucuronide in samples was calculated based on a standard curve utilizing mirtazapine due to the lack of availability of 8-hydroxymirtazapine for use as a standard. 8-hydroxymirtazapine values were obtained from the saccharic acid treated samples and the amount of 8-hydroxymirtazapine glucuronide calculated as the amount in β-glucuronidase minus saccharic acid treated samples.

***4.3.1g. Pharmacokinetic Analysis.***

Pharmacokinetic analysis was performed using a non-compartmental method. Area under the curve to infinity ($AUC_\infty$), disappearance half-life ($t_{1/2}\lambda$), time to maximum serum concentration ($T_{max}$), and maximum serum concentration ($C_{max}$) were calculated. Because mirtazapine was administered via an extravascular route, absorbed dose is equal to D (dose) x bioavailability (F), and thus the estimation of parameters in which the calculation is based on the assumption that 100% of the dose reaches systemic circulation (clearance [CL] and volume of distribution [Vd]) are represented as clearance corrected for bioavailability or clearance/bioavailability (CL) and volume of distribution/bioavailability (Vd/F).[14] Using the term CL and comparing it between the two treatment groups assumes that F, or bioavailability, is not different between the two treatment groups. The accumulation factor at steady state following multiple doses was estimated from the pharmacokinetic data using the equation: Accumulation Factor =

$1/1\text{-e-}k_{el}*T$. The terminal elimination rate was used for estimating the accumulation factor as the applicable $k_{el}$ and the dosing interval (T) was set at 24 or 48 hours.

***4.3.1h. Statistical Analysis.***

Comparison of the pharmacokinetic parameters between HD and LD groups were performed with Mann-Whitney statistical analysis. For all analyses, a p-value of $< 0.05$ was considered to be statistically significant.

### 4.3.2 Results

#### *4.3.2a Cats*

*Normal Cats*

Ten young adult healthy cats with normal physical examination, serum biochemistry profile, complete blood count and urinalysis were entered in the study. There were 4 neutered males and 6 spayed females. Breeds included 9 domestic shorthairs and one domestic longhair. The median age was 2.3 years with a range of 1.4 to 4.1 years, and the mean weight was 5.3 with a range of 3.9 to 7.8 kg. Five cats received a single oral dose of 3.75 mg mirtazapine (HD) (dose range 0.48-0.73 mg/kg; median 0.62 mg/kg) and five cats received a single oral dose of 1.88 mg mirtazapine (LD) (dose range 0.35-0.48 mg/kg; median 0.44 mg/kg). A summary of demographics and dosing is presented in **Table 4.1**.

**Table 4.1:** Summary of demographic data for normal cats by study.

| | Age (years) | | Weight (kg) | | | Dose (mg/kg) | |
|---|---|---|---|---|---|---|---|
| Study type | Median | Range | Median | Range | Dose group | Median | Range |
| PK single dose ($n$ = 10 cats) | 2.3 | 1.4–4.1 | 5.3 | 3.9–7.8 | LD | 0.44 | 0.35–0.48 |
| | | | | | HD | 0.62 | 0.48–0.73 |
| Daily multiple dosing ($n$ = 4 cats) | 1.4 | 1.4–1.4 | 5.1 | 3.87–6.45 | LD | 0.37 | 0.29–0.49 |
| | | | | | HD | N/A | N/A |

Comparison of the mg/kg dose of mirtazapine between the HD and LD groups, using a Mann-Whitney test, demonstrated that the doses were significantly different ($p = 0.015$). No cats experienced adverse effects from drug administration although increased activity, social interaction and vocalization were noted subjectively in nine cats (objective assessment was performed in the pharmacodynamic portion of the project).

*CKD Cats and Age-Matched Control Cats*

Six client-owned stable CKD cats (two each from IRIS stages II, III and IV) and 6 AMC cats were enrolled. There were 4 spayed females and 2 neutered males in the CKD group and 3 spayed females and 3 neutered males in the AMC group. Breeds included 4 DSH, 1 DLH and 1 Himalayan in the CKD group and 4 DSH and 2 DLH in the AMC group. The mean age ± standard deviation (SD) of the CKD group was 11 ± 2.2 years (range 8.3 – 13.7 years; median 11 years) and the mean age ± SD of the AMC group was 10.8 ± 2.3 years (range 7.8 – 13.8 years; median 10.7 years). The mean mg/kg dose ± SD of mirtazapine in the CKD group was 0.51 ± 0.15 mg/kg (range 0.4 – 0.78 mg/kg; median 0.45 mg/kg) and the mean mg/kg dose ± SD of mirtazapine in the AMC group was 0.44 ± 0.08 mg/kg (range 0.33 – 0.58 mg/kg; median 0.43 mg/kg). There was no statistically significant difference in age or mg/kg dose between the two groups ($p = 0.93$ and $p = 0.57$ respectively). The mean creatinine ± SD in the CKD group was 3.8 ± 1.6 mg/dL (range 2.4 – 6.1 mg/dL; median 3.1 mg/dL) and the mean creatinine ± SD in the AMC group was 1.3 ± 0.4 mg/dL (range 0.7 – 1.8 mg/dL; median 1.4 mg/dL). Specifically, 2 cats in IRIS Stage II (creatinine 2.4 and 2.5 mg/dL), 2 cats in IRIS Stage III (creatinine 2.9 and 3.3 mg/dL) and 2 cats in IRIS Stage IV (5.7 and 6.1 mg/dL) were enrolled in the CKD group. A summary of demographics and dosing is presented in **Table 4.2.**

**Table 4.2:** Descriptive statistics of AMC cats (n=6) and CKD cats (n=6). No statistically significant difference was found in age or mg/kg dose between the groups.

| **Descriptive Statistics of Enrolled Healthy Geriatric Cats and Cats with Chronic Kidney Disease** | | | | | | |
|---|---|---|---|---|---|---|
| **Pharmacokinetic Parameter** | **Healthy Geriatric** | | | **Chronic kidney disease** | | |
| | **Median** | **Range** | **Mean ± SD** | **Median** | **Range** | **Mean ± SD** |
| **Age (years)** | 10.7 | 7.8 – 13.8 | 10.8 ± 2.3 | 11 | 8.3 – 13.7 | 11 ± 2.2 |
| **Mg/kg dose** | 0.43 | 0.33 – 0.58 | 0.44 ± 0.08 | 0.45 | 0.4-0.78 | 0.51 ± 0.15 |
| **Creatinine (mg/dL)** | 1.4 | 0.7 – 1.8 | 1.3 ± 0.4 | 3.1 | 2.4 – 6.1 | 3.8 ± 1.6 |

### *4.3.2b Pharmacokinetic Study*

*Normal Cats*

Results for normal cat dose comparison pharmacokinetics and are shown in **Table 4.3**. Overall there was a statistically significant difference between the elimination half-life, clearance, AUC/Dose, and $AUC_{\infty}$/Dose of the LD and HD groups when they were compared with Mann-Whitney nonparametric statistics ($p = 0.03$ for all comparisons). Drug concentration curves for normal cats in the HD and LD comparison groups are shown in **Figure 4.1**.

**Table 4.3:** Pharmacokinetic parameters comparing two doses of orally-dosed mirtazapine in healthy young cats.*Significantly different ($p < 0.05$) values between LD and HD groups as determined by a Mann-Whitney test.

| | High dose (HD) (3.75 mg) | | | Low dose (1.88 mg) | | |
|---|---|---|---|---|---|---|
| Pharmacokinetic parameter | Median | Range | Mean ± SD | Median | Range | Mean ± SD |
| $C_{max}$ (ng/mL) | 183.9 | 40.1–272.8 | 156.5 ± 92.4 | 45.3 | 34.1–136.9 | 73.1 ± 45.5 |
| $C_{max}$/Dose (ng/mL)/(mg/kg) | 250.6 | 82.9–399.4 | 243 ± 132 | 126.2 | 77.9–281.9 | 170 ± 96 |
| $T_{max}$ (h) | 1 | 0.5–4 | 1.5 ± 1.4 | 1 | 1–4 | 1.6 ± 1.3 |
| Half-life (h)* | 15.9 | 11.0–21.8 | 15.3 ± 4.7 | 9.2 | 8.7–14.3 | 10.3 ± 2.3 |
| AUC (ng/mL·h) | 942.9 | 443.8–1458.6 | 990.2 ± 372.4 | 364.9 | 289.4–562.0 | 397 ± 105.6 |
| AUC/Dose (ng/mL·h)/(mg/kg)* | 1622 | 917–2135 | 1567 ± 483 | 830 | 771–1157 | 926 ± 180 |
| $AUC_{\infty}$ (ng/mL·h) | 1047.9 | 501.8–1498.4 | 1088.6 ± 400.1 | 376.1 | 310.9–570 | 407.4 ± 102.1 |
| $AUC_{\infty}$/Dose (ng/mL·h)/(mg/kg)* | 1670.2 | 1037.1–2297.3 | 1725 ± 527 | 891 | 816–1173 | 953 ± 173 |
| CL/F (L/h/kg)* | 0.59 | 0.43–0.96 | 0.63 ± 0.22 | 1.12 | 0.85–1.27 | 1.08 ± 0.19 |
| $Vd_{\beta}$/F (L) | 13.7 | 6.9–22.4 | 14.1 ± 6.2 | 16 | 11.3–23.2 | 16.2 ± 4.9 |

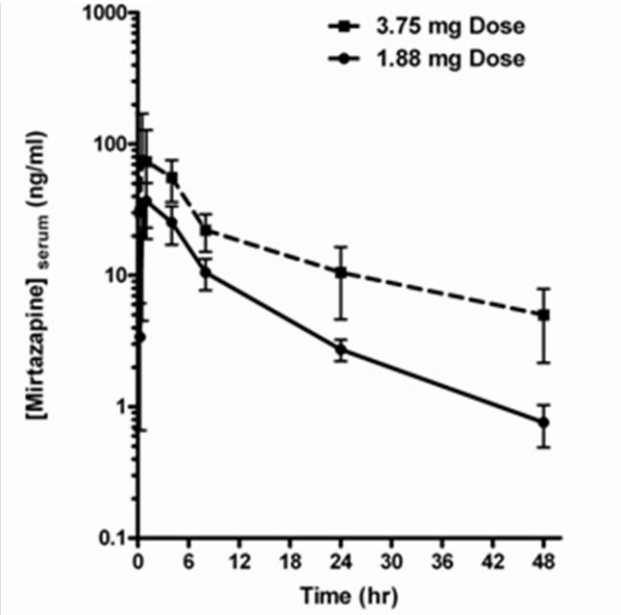



**Figure 4.1:** Drug concentration curves for cats in the HD and LD groups. Note that the lines deviate, which represents a statistically significant difference in elimination half-life (p = 0.03) between doses.

*Normal Cat Metabolite Analysis*

Metabolite analysis revealed that measurable quantities of 8-hydroxymirtazapine, the first product produced by Phase 1, were detected (**Figure 4.2**). Mean AUC was 4.09 ± 0.54 ng/mL • h (HD) and 2.63 ± 0.68 ng/mL • h (LD). Thus there was a trend towards higher values in the HD group, but this did not reach statistical significance (p = 0.08). The amount of metabolite undergoing glucuronidation was also measurable and the mean AUC was 11.72 ± 2.53 ng/mL • h (HD) and 17.3 ± 6.44 ng/mL • h (LD), suggesting the same or slightly lower concentration in the HD group compared to the LD group (**Figure 4.3**). This difference was not statistically different (p = 0.19).

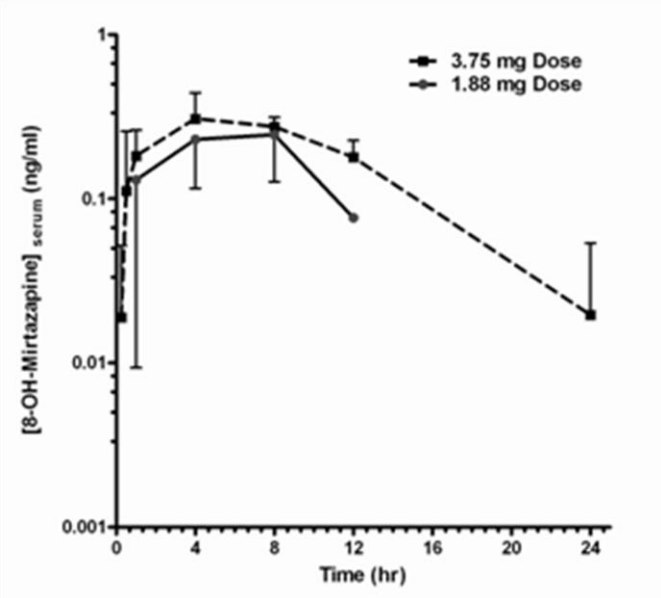



**Figure 4.2:** 8-Hydroxymirtazapine metabolite concentrations. 8-Hydroxymirtazapine appears to be present in higher concentrations in the HD group, although the difference was not statistically significant (p=0.08).

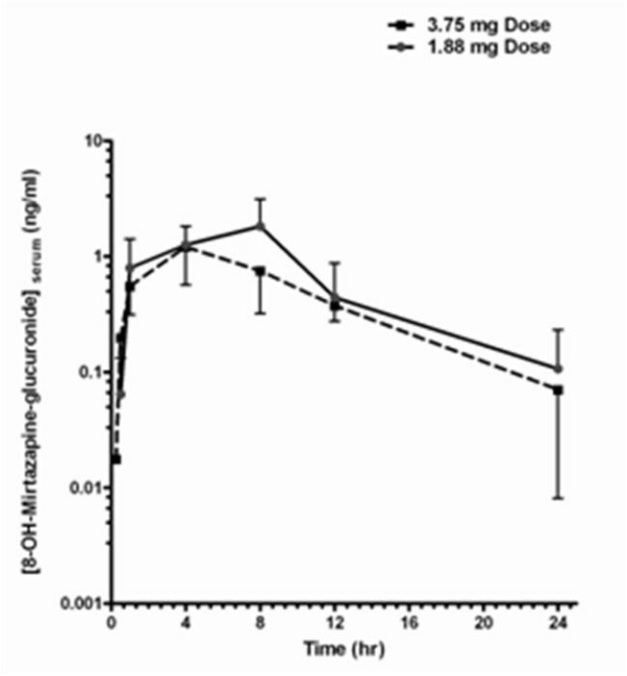



**Figure 4.3:** Glucuronidated metabolite concentrations. The glucuronidated metabolite is present in essentially the same quantities in the LD and HD groups (p = 0.19).

*CKD Cats and Age-Matched Controls*

Pharmacokinetic parameters for all three groups at the low dose are shown in **Table 4.4**. There was a statistically significant difference in $AUC_{\infty}$ and CL between the AMC cats and CKD cats. Graphical representations of drug concentration curves for all three groups are illustrated in **Figure 4.4.**

**Table 4.4** Pharmacokinetic parameters for orally-dosed mirtazapine in young healthy, CKD and AMC cats. *Significantly different ($p < 0.05$) values between AMC and CKD groups as determined by a Mann-Whitney test.

| Pharmacokinetic Parameters of Orally-Dosed Mirtazapine in Young Healthy Cats, Healthy Geriatric Cats and Cats with Chronic Kidney Disease | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Pharmacokinetic Parameter** | **Young Healthy**** | | | **Healthy Geriatric** | | | **Chronic kidney disease** | | |
| | **Median** | **Range** | **Mean ± SD** | **Median** | **Range** | **Mean ± SD** | **Median** | **Range** | **Mean ± SD** |
| **$C_{max}$ (ng/mL)** | 45.3 | 34.1 - 136.9 | 73.1 ± 45.5 | 83.6 | 50.2 - 103 | 79.6 ± 21.7 | 109.5 | 79.1 - 164 | 110.6 ± 30.8 |
| **$T_{max}$ (hr)** | 1 | 1 - 4 | 1.6 ± 1.3 | 1 | 1 - 4 | 2 ± 1.5 | 1 | 0.5 – 1.5 | 1 ± 0.3 |
| **Half life (hr)** | 9.2 | 8.7 - 14.3 | 10.3 ± 2.3 | 12.0 | 10.1 – 15.4 | 12.3 ± 1.8 | 15.8 | 10.8 – 24.8 | 16.4 ± 5.1 |
| **AUC * (ng/mL • hr)** | 364.9 | 289.4 - 562.0 | 397 ± 105.6 | 523.9 | 384.7 – 894.3 | 555.5 ± 175.4 | 686.5 | 567.3 – 1147.5 | 770.6 ± 225.5 |
| **$AUC_{\infty}$ * (ng/mL • hr)** | 376.1 | 310.9 - 570 | 407.4 ± 102.1 | 560.8 | 400.4 – 941.2 | 589.8 ± 185.3 | 828.4 | 597.1 – 1253.6 | 866.5 ± 257.9 |
| **CL/F * (L/hr/kg)** | 1.12 | 0.85 - 1.27 | 1.08 ± 0.19 | 0.73 | 0.62 – 1.1 | 0.79 ± 0.16 | 0.6 | 0.45 – 0.74 | 0.61 ± 0.1 |
| **$Vd_{\beta}/F$ (L)** | 16 | 11.3 - 23.2 | 16.2 ± 4.9 | 14.4 | 10.1 – 16.3 | 13.9 ± 3.2 | 13.4 | 11.5 – 16.1 | 13.6 ± 2.0 |

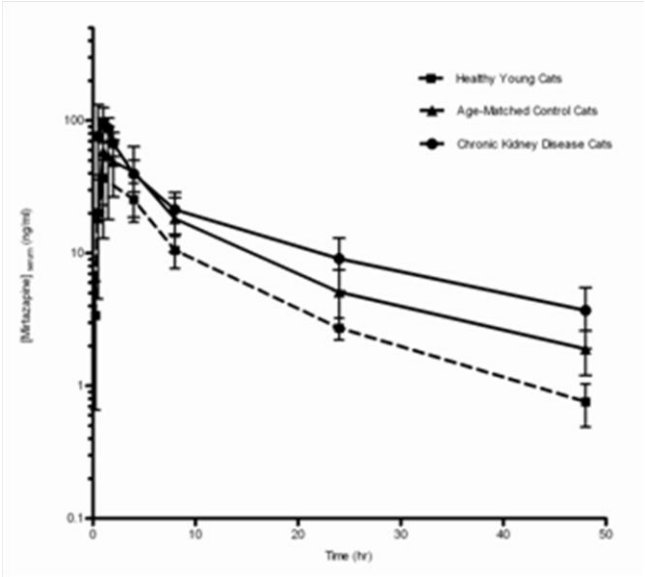



**Figure 4.4:** Drug concentration curves for mirtazapine in CKD cats and AMC cats in comparison to data from young healthy cats. Age appears to affect the metabolism of the drug, but does not entirely explain the increased exposure in cats with CKD.

*Relationship Between Serum Creatinine and Mirtazapine Clearance*

There was a significant negative correlation between serum creatinine and clearance of mirtazapine ($r = -0.69$ with $p = 0.0024$) when data from young normal cats, normal geriatric cats and CKD cats were analyzed. This relationship is depicted in **Figure 4.5.**

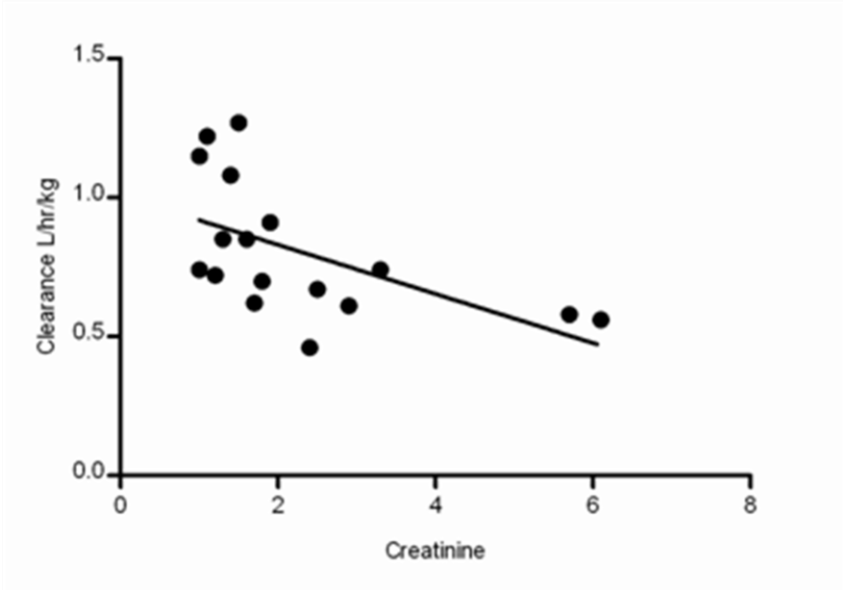



**Figure 4.5:** Relationship between serum creatinine and clearance of mirtazapine. Spearman Rank analysis revealed a significant negative correlation ($r = -0.69$) with $p = 0.0024$. As kidney disease progresses, clearance of mirtazapine decreases.

### *4.3.2c Daily Administration and Accumulation*

Four cats (2 female, 2 male) received LD mirtazapine orally once daily for 6 days. Calculation of drug accumulation from the pharmacokinetic data indicated that minimal accumulation (AF = 1.2) was expected with daily administration of the LD. A median Day 3 trough level of 1.4 ng/mL (range: 0.22 – 3.36 ng/mL), and a median 2 hour post-administration drug concentration of 38.6 ± 0.2 ng/mL (range: 38.4-38.8 ng/mL) was observed. On Day 6 the median trough level was 1.53 ng/mL (range: 0 – 3.27 ng/mL), and the median 2 hour post-administration drug concentration was 29.5 ng/mL (range: 24.7 – 54.8 ng/mL). These concentrations were similar to the range measured in the pharmacokinetic curve at the corresponding timepoint (range 25.2-80.2 ng/mL; median 36.8 ng/mL). No evidence of drug accumulation was detected. Subjectively, no cats experienced changes in activity, social interaction and vocalization during the 6 day time period (objective assessment was performed in the pharmacodynamic study as described below).

Drug accumulation was calculated for 24- and 48-hour dosing intervals for both CKD and AMC groups based on the available pharmacokinetic data. For the CKD cats, an accumulation factor of 1.57 was calculated for 24-hour dosing and an accumulation factor of 1.15 was calculated for 48-hour dosing. For the AMC cats, an accumulation factor of 1.35 was calculated for 24-hour dosing and an accumulation factor of 1.07 was calculated for 48-hour dosing. This is in comparison to the calculated accumulation factor of 1.2 for 24-hour dosing in young normal cats.

## 4.4 Pharmacodynamics of Mirtazapine in Normal Cats

### 4.4.1 Materials and Methods

#### *4.4.1a. Cats*

Fourteen privately owned young adult healthy cats were enrolled in the pharmacodynamic study after undergoing a physical examination, complete blood count, serum biochemistry profile and urinalysis to rule out systemic disease.

#### *4.4.1b. Study Design*

This was a randomized, double-blind, placebo-controlled crossover study. Cats were fasted for 12 hours before beginning the study. All fourteen cats received placebo, 1.88 mg and 3.75 mg of mirtazapine on three separate occasions, one week apart in a predetermined random sequence. Placebo and both mirtazapine doses were formulated into identical capsules that were administered orally, followed by 3 mL of water administered by syringe. Before capsule administration a baseline behavioral assessment was made (see appendix 1; the behavioral scores from each observation over the eight hour period were tallied to give the total behavior score for that particular treatment). One hour after medication administration, the cats received a behavioral assessment and one cup of their usual dry diet, which was weighed on a gram scale (ZL200630016361.1, China), the accuracy of which was assessed by measuring National Institute of Standards and Technology traceable calibration weights. At 1 hour intervals for an eight hour period, the cats' remaining food was weighed and a behavioral assessment was made by a single blinded observer. Blood samples were obtained 2 and 8 hours after capsule administration for analysis of mirtazapine serum concentration; samples were handled as previously described. The cats were released to their owners nine hours after mirtazapine administration. Owners were asked to complete a home assessment questionnaire including perceived changes in appetite, vocalization and activity the evening of, and the morning after, the study.

#### *4.4.1c. Mirtazapine Analysis*

Mirtazapine analysis was performed as previously described.

#### *4.4.1d. Pharmacokinetic Analysis*

The data from the initial pharmacokinetic time course study were utilized to develop a limited-sampling approach for pharmacokinetic analysis during the pharmacodynamic study. Analysis of the AUC data versus specific time point values from the 5 HD and 5 LD animals was done by multiple linear regression (Minitab v15.1.1.0, Minitab Inc., State College PA) to determine if one to three samples could accurately predict the total AUC value.[15] The AUC versus the 8 hour time point resulted in the best prediction with a $r^2$=0.909. Therefore, the AUC for the pharmacodynamic portion of the studies was estimated based on the value of the 8 hour time point utilizing the equation AUC (ng/ml x hr) = -21.4 + 28.6 x (8 hour time point (ng/ml)).

#### *4.4.1e. Statistical Analysis.*

Comparison of percentage of food ingested and the total behavioral scores for each day during the pharmacodynamic study was performed with repeated measures ANOVA and Bonferroni post-hoc analysis. Prism software (GraphPad Software, Inc, La Jolla, CA) was used for analysis. Comparison of at home assessment parameters was performed using a mixed-effects logistic regression model[16] with SAS software (SAS, version 9.2, SAS Institute Inc., Cary, N.C). Cat identity was nested within treatment sequence (the primary exposure of interest) to account for random and repeated effects. Treatment sequence was included as a random effect in each model, regardless of whether a significant association could be identified when treatment sequence was analyzed as a fixed effect. This method conservatively accounts for possible incomplete washout, even though this was not expected.[16] For all analyses, a p-value of $< 0.05$ was considered to be statistically significant.

### 4.4.2 Results

#### *4.4.2a Cats*

Fourteen young adult healthy cats with normal physical examination, serum biochemistry profile, complete blood count and urinalysis were entered in the study. Breeds included 7 domestic shorthairs, 4 domestic longhairs, 2 siamese mixes and 1 Burmese. Eight cats were spayed females and 6 cats were neutered males. The median age was 3.3 years (range 1.0-4.5 years) and the median weight was 4.2 kg (range 2.9-6.8 kg). When LD mirtazapine was administered, the median dose was 0.45 mg/kg (range 0.27-0.64 mg/kg). When HD mirtazapine was administered, the median dose was 0.89 mg/kg (range 0.56-1.27 mg/kg).

#### *4.4.2b Pharmacodynamics*

The effect of mirtazapine administration on food consumption is illustrated in **Figure 4.6**. There was a statistically significant difference ($p = < 0.0001$) between the amount of food eaten after administration of either LD or HD mirtazapine in comparison to placebo. Cats ate an average of 53.2% of their food when receiving the LD, and 56.7% of their food when receiving the HD. This was in comparison to eating 13% of their food when receiving the placebo. There was no significant difference in the amount of food consumed between the two doses of mirtazapine. Nine of 14 cats refused food entirely when administered placebo and 8/9 of these cats ate after mirtazapine was administered at either dose. One cat refused food entirely regardless of treatment administered.

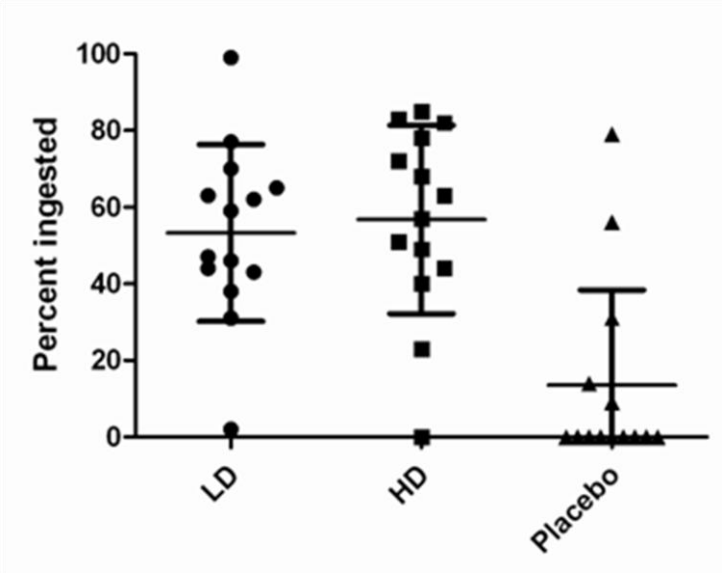



**Figure 4.6** Effect of administration of LD mirtazapine, HD mirtazapine or placebo on food consumption. There is a statistically significant difference in the amount of food ingested between placebo and either LD or HD mirtazapine ($p < 0.0001$). There is no significant difference in the amount of food ingested between mirtazapine doses.

The effect of mirtazapine administration on interaction, activity level and vocalization scores is illustrated in **Figures 4.7-4.9**. There was a statistically significant difference between administration of either LD or HD mirtazapine in comparison to placebo for all three parameters (interaction $p < 0.0001$; activity level $p < 0.0001$; vocalization $p = 0.0028$). Bonferroni's post-hoc analysis determined that there was a statistically significant difference ($p < 0.05$) between the LD and HD for interaction and vocalization scores, but not for activity level ( 95% confidence interval: – 5.7 to – 0.15 for interaction and – 5 to – 0.7 for vocalization).

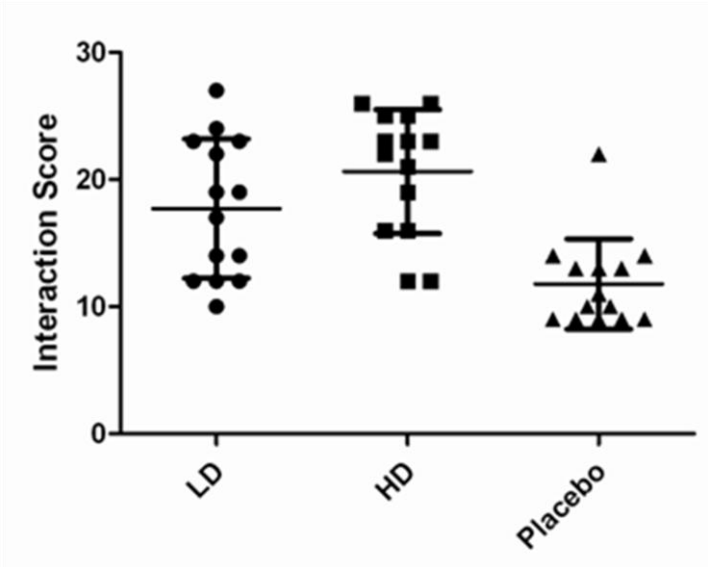



**Figure 4.7**: Effect of administration of LD, HD or placebo on interaction scores. There is a statistically significant difference in interaction scores between placebo and either LD or HD mirtazapine ($p < 0.0001$). There is also a statistically significant difference in interaction scores between administration of LD or HD ($p < 0.05$).

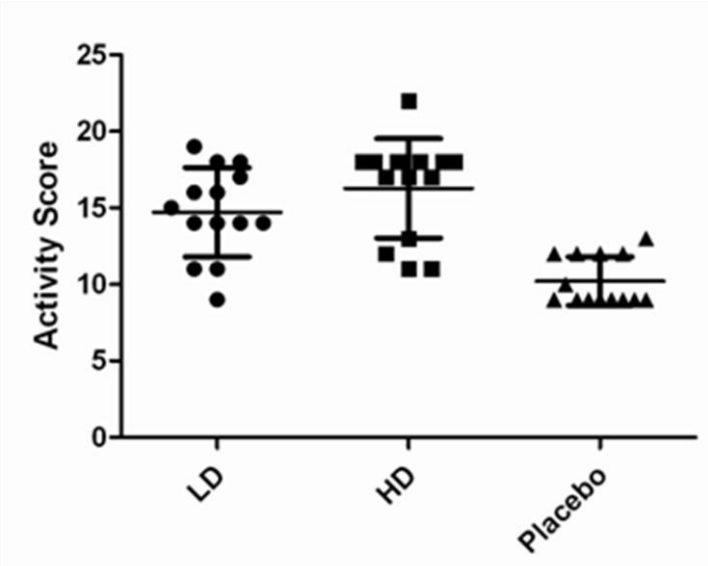



**Figure 4.8:** Effect of administration of LD, HD or placebo on activity level scores. There is a statistically significant difference in activity level scores between placebo and either LD or HD mirtazapine ($p < 0.0001$). There is no significant difference in activity level scores between mirtazapine doses.

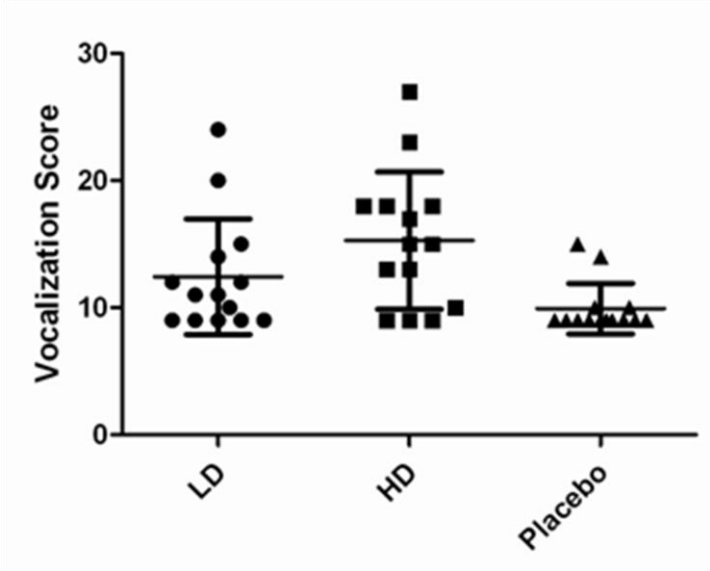



**Figure 4.9:** Effect of administration of LD, HD or placebo on vocalization scores. There is a statistically significant difference in vocalization scores between placebo and either LD or HD mirtazapine ($p < 0.0001$). There is also a statistically significant difference in vocalization scores between administration of LD or HD ($p < 0.05$).

Cats were also assessed for the presence of tremors or twitching and no events were recorded during the eight hour observation period. At-home owner assessment of the effect of mirtazapine on appetite, activity and vocalization is illustrated in **Table 4.5**.

**Table 4.5:** At-home owner assessment of appetite, activity and vocalization after mirtazapine administration.

| | | Appetite | | | Vocalization | | | Activity | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Placebo | HD | LD | Placebo | HD | LD | Placebo | HD | LD |
| 10–14 h after administration | Increased | 1/14 | 6/14 | 9/14 | 1/14 | 7/14 | 5/14 | 1/14 | 6/14 | 4/14 |
| | Normal or decreased | 13/14 | 8/14 | 5/14 | 13/14 | 7/14 | 9/14 | 13/14 | 8/14 | 10/14 |
| 20–24 h after administration | Increased | 0/14 | 2/14 | 4/14 | 0/14 | 3/14 | 2/14 | 1/14 | 3/14 | 4/14 |
| | Normal or decreased | 14/14 | 12/14 | 10/14 | 14/14 | 11/14 | 12/14 | 13/14 | 11/14 | 10/14 |

HD, high dose; LD, low dose.

Using mixed-effects logistic regression, it was determined that cats receiving mirtazapine still had a significantly increased appetite approximately 12 hours after drug administration when compared to cats that received placebo ($p = 0.03$), however there was no significant difference in appetite 24 hours after

drug administration ($p = 0.18$). No statistically significant difference was seen for vocalization at either timepoint (12 hours: $p = 0.05$, 24 hours: $p = 0.3$) or activity at either timepoint (12 hours: $p = 0.15$, 24 hours: $p = 0.33$).

#### *4.4.2c Estimation of AUC Utilizing a Limited Sampling Approach*

A limited sampling approach was utilized to estimate the AUC of mirtazapine in the cats for the pharmacodynamic studies.[15] The $AUC_{0\text{-}24}$ estimated in the pharmacodynamic cats using this limited sampling model were 440.2 ± 137.5 and 1073.3 ± 414.4 ng/ml x hr for the LD and HD, respectively. These values were not significantly different from those calculated in the full course pharmacokinetic study at these same doses and show essentially the same ratio in exposure between the HD and LD groups. No correlation between dose and effect was found when drug exposure, as represented by AUC, was compared to amount of food eaten ($r = 0.53$), vocalization ($r = 0.50$), activity ($r = 0.64$) and interaction ($r = 0.53$).

## 4.5 Pharmacodynamics of Mirtazapine in Cats with Chronic Kidney Disease: Clinical Trial

### 4.5.1 Materials and Methods

#### *4.5.1a Cats*

11 client-owned cats with stable CKD (serum creatinine of 2.0 – 6.0 mg/dl) and history of decreased appetite and/or poor body condition were enrolled. Diagnostic tests required before enrollment included a serum biochemistry profile, complete blood count, urinalysis, urine culture, blood pressure, and serum total thyroxine measurement. Exclusion criteria include other systemic illnesses, complications of CKD such as pyelonephritis or ureteral obstruction, or decompensation of CKD requiring hospitalization and intravenous fluid therapy. Other concurrent therapies such as dietary management, famotidine, potassium supplementation, anti-hypertensive medications and subcutaneous fluids were accepted therapies if they were started more than 2 weeks before the beginning of the trial and given consistently throughout the study period. No treatment changes were allowed during the study period; if treatment changes were deemed medically necessary, the cat was removed from the study.

#### *4.5.1b Study Design*

This was a double blind placebo-controlled prospective study. Mirtazapine was compounded into a 1.88 mg (1/8 of the commercially available 15 mg tablet) capsule by the CSU Veterinary Medical Center pharmacy according to Professional Compounding Centers of America® protocol. The method used is guaranteed to produce accurate compounding to within 10% of the target dose. An identical placebo capsule was manufactured containing lactose. The lots were coded A and B and the pharmacy staff kept the key to the code. A preset randomization for order of distribution (AB or BA) was determined and as cats were enrolled they were assigned consecutively to a treatment regime. The clinician and owner were masked as to the treatment order. A serum biochemistry profile was performed prior to entry. The first treatment was given every 48 hours for three weeks followed by a 4-day (equivalent to 5 half-lives) washout period before the second treatment was given on the same dosing schedule. Owners were asked

to fill out a daily log sheet regarding their cats' behavior, appetite, demeanor, and number of vomiting episodes and this information was later converted to clinical scores. At the end of each phase, the log sheets were collected and the cats were weighed and examined and a serum biochemistry profile was performed. For cats participating at ancillary veterinary clinics, serum samples were shipped to Colorado State University for analysis.

#### *4.5.1c Statistical Analysis*

In cats with CKD, an increase in body weight is a readily obtained objective measure of increased food intake. Other parameters to be assessed in this study were subjective (such as client assessment of appetite and activity level). Differences in weight, body condition scores, clinical scores, and vomiting episodes were compared using a Wilcoxon sign rank test. All analyses were performed using Prism software. For all analyses, a P-value of $<0.05$ was considered to be statistically significant.

### 4.5.2 Results

#### *4.5.2a Cats*

A total of 14 cats with CKD were enrolled in the clinical trial. Eleven cats successfully completed the trial. One cat was enrolled but did not start the study, and two cats were disqualified for uremic crises during the study; one during the placebo phase and one during the mirtazapine phase. Of the 11 cats that completed the trial, there were 5 domestic shorthairs, 3 Siamese mixes, 1 Tonkinese, 1 Persian and 1 domestic longhair. The median age was 15 years (range 10-17). Seven cats were classified as IRIS stage II, three cats were IRIS stage III and 1 cat was IRIS stage IV.

### *4.5.2b Effect of Mirtazapine on CKD Cats*

The administration of mirtazapine for three weeks to cats with CKD resulted in a statistically significant increase in weight ($p = 0.002$; **Figure 4.10**), appetite score ($p = 0.02$; **Figure 4.11**), activity score ($p = 0.02$; **Figure 4.12**) compared to three weeks of administration of placebo. Weight gain was seen in 91% of the CKD cats administered mirtazapine, and in contrast 82% of cats lost weight during the placebo phase. Median weight gain during mirtazapine administration was 0.18 kg (range 0-0.45 kg). Median weight loss during placebo administration was 0.07 kg (range 0-0.34 kg). Weight loss during the placebo phase was also statistically significant ($p = 0.02$). Ninety-one % of cats had increased appetite score on mirtazapine and 55% had an increased activity score. Additionally there was a statistically significant decrease in vomiting ($p = 0.047$; **Figure 4.13**) when mirtazapine was administered. A statistical trend was seen in increase in body condition score ($p = 0.053$) with 45% of cats experiencing an increase in body condition. All of the cats who experienced increased body condition had suboptimal body score, whereas 85% of those that had no change already had an optimal body score. No owners reported adverse behavioral changes associated with mirtazapine administration.

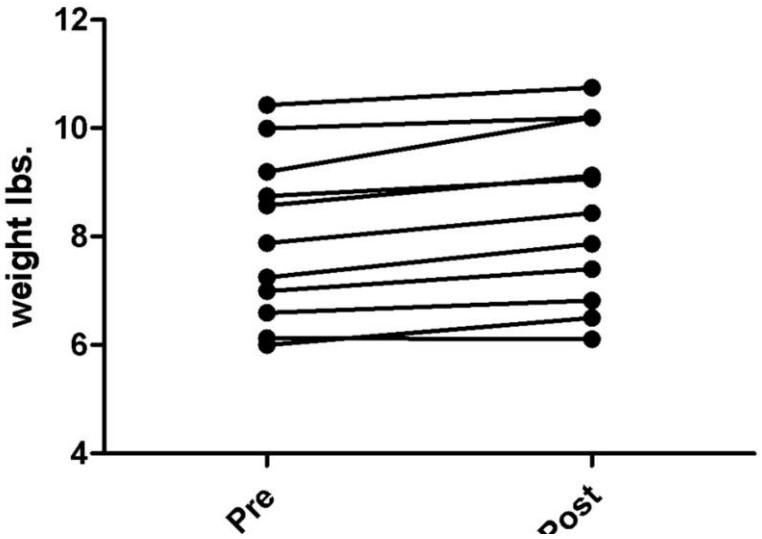



**Figure 4.10**: Effect of mirtazapine on weight. A statistically significant increase in weight was seen in CKD cats administered 1.88 mg mirtazapine every other day for three weeks (p = 0.002).

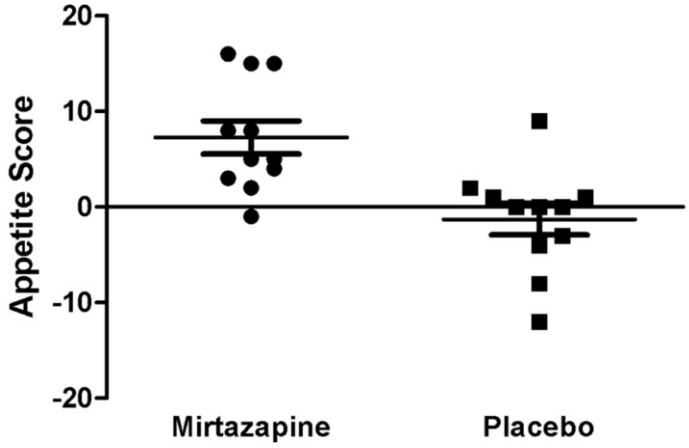



**Figure 4.11:** Effect of mirtazapine on appetite. A statistically significant increase in appetite score was seen in CKD cats administered 1.88 mg mirtazapine every other day for three weeks (p = 0.02).

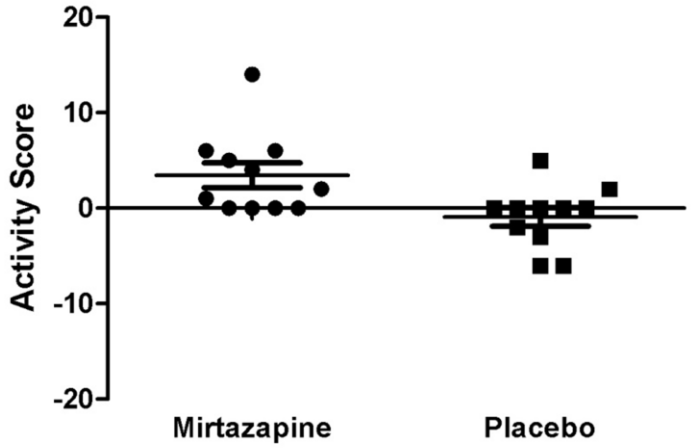



**Figure 4.12:** Effect of mirtazapine on activity. A statistically significant increase in activity was seen in CKD cats administered 1.88 mg mirtazapine every other day for three weeks (p = 0.02).

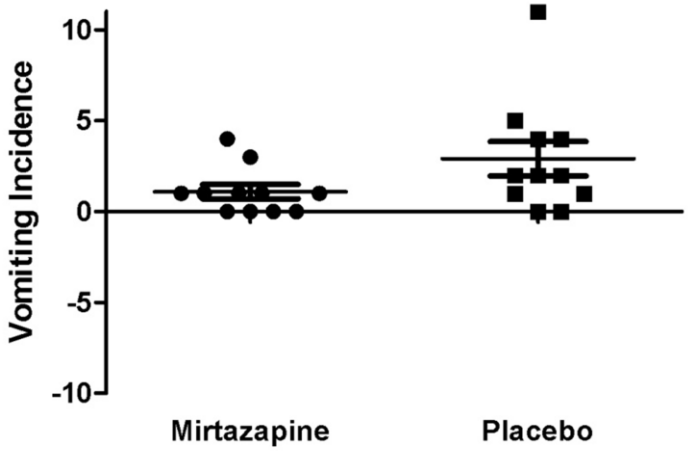



**Figure 4.13:** Effect of mirtazapine on vomiting. A statistically significant decrease in vomiting was seen in CKD cats administered 1.88 mg mirtazapine every other day for three weeks (p = 0.047).

Serum biochemistry values were performed before starting the study, between treatment phases and at the end of the study. No statistically significant differences in creatinine, potassium or other biochemical parameters were seen as a result of mirtazapine administration. One cat experienced a marked elevation in alanine aminotransferase (ALT) of 744 mg/dL with a normal alkaline phosphatase, presumably as a result of the three weeks of mirtazapine administration, with no associated clinical abnormalities. This elevation resolved within three weeks, once mirtazapine was discontinued. After the end of the study, the owner elected to once again try mirtazapine therapy as the cat had clinically responded so well. ALT elevation (731 mg/dL) with no associated clinical signs was once again noted and once again resolved within three weeks after the discontinuation of mirtazapine.

## 4.6 Discussion

The efficacy of mirtazapine as an appetite stimulant in cats is supported by the results of this body of work, which demonstrated a significant increase in appetite after administration of the drug to young normal cats and cats with CKD. In the normal cat pharmacodynamic study, there was a significant increase in food consumption when mirtazapine was administered at either dose. Eighty-eight percent of healthy cats that refused to eat their normal diet while in the hospital subsequently ate when mirtazapine was administered. No significant difference in the amount of food consumed was seen between the two doses, however a significant increase in behavior such as vocalization and interaction was noted at the higher dose. The implication of these findings is that similar efficacy with less potential for side effects is experienced with the lower dose and this may be the most appropriate initial dose for many cats, particularly those with renal compromise. Subsequently, in the CKD cat clinical trial, all cats tolerated low dose mirtazapine with minimal side effects and a significant increase in weight, appetite and activity was seen, as well as a significant decrease in vomiting.

The data gathered in the pharmacokinetic studies suggests that the dosing interval in cats is significantly different from that previously postulated.[11] The half-life in humans ranges from 20 to 40 hours and the drug is administered daily.[10] In contrast, the half-life of mirtazapine in young normal cats at

the lowest effective dose (1.88mg) is approximately 10 hours in young normal cats, 12 hours for healthy geriatric cats and 15 hours for CKD cats. Based on this half-life, the drug could theoretically be administered daily in normal cats and every other day in CKD cats without substantial drug accumulation. This is supported by the daily dosing data that was collected. Although only a small number of cats were used, and therefore the data were only subjectively described, 24 hour trough levels were close to the limits of detection and no accumulation was seen after daily dosing for 6 days. Serum levels may not necessarily relate to drug effect in cats. No concentration-effect relationship has been found in humans treated with mirtazapine, and when used at therapeutic doses, the serum concentration has been reported to range from 5-100 μg/L.[10] The $C_{max}$ is reported to range from 56.1 to 74.5 μg/L in healthy human males[10] and this is similar to the range found in the feline study patients.

Dose proportionality is exhibited with mirtazapine in human pharmacokinetic studies,[10] and regardless of the dose administered, the elimination half-life remains the same. This does not appear to be true in cats, based on the data collected in this study, as there was a significant difference between the elimination half-lives of the HD and LD groups. The pharmacokinetic study was not a crossover study in design and this may have potentially affected the data and interpretation. However a significant difference in drug exposure and clearance does appear to exist between the dose groups. The results of the glucuronide metabolite data may provide some explanation for this observation. The accumulation of higher levels of the glucuronide metabolite might have been predicted in the HD group, as there were slightly higher levels of 8-hydroxymirtazapine detected in that group. However there was essentially the same amount, if not less, of glucuronide metabolite present in the HD group. Possible explanations for this result include saturation of the glucuronide enzyme system, which is known to be deficient in cats,[12] or inhibition of metabolism by the 8-hydroxymirtazapine metabolite. This would be concerning not only because of a potential for more drug side effects, but also because depletion of glucuronidation ability could affect metabolism and serum concentrations of other drugs and supplements. These findings suggest that it may be more appropriate to administer the 1.88 mg dose of mirtazapine, in order to avoid these potential effects on drug metabolism.

A significant difference in drug exposure (AUC) and clearance of mirtazapine was found between CKD cats and AMC cats. The mean AUC in normal cats was 397 ng/mL/hr, in comparison to 523.9 ng/mL/hr in AMC cats and 686.5 ng/mL/hr in CKD cats. Although there was no significant difference in mirtazapine dose between the CKD and AMC cats, the statistical power of the comparison between the doses in each group is likely limited due to sample size. Therefore AUC and $C_{max}$ were also calculated with dose adjustment (**Table 2**) to diminish the possible effect of difference in dose between the two groups. Even with this adjustment, AUC was still significantly different between the AMC and CKD groups. The mean CL was found to be 1.1 L/hr/kg in young healthy cats, in comparison to 0.79 L/hr/kg in AMC cats and 0.61 L/hr/kg in CKD cats. From this information, we suggest that although age appears to have some influence on the metabolism of the drug, it cannot entirely account for the difference between CKD and young normal cats. Therefore we conclude that CKD delays the CL of mirtazapine in cats.

In the human literature, it is reported that moderate to severe renal disease results in increased mirtazapine exposure (AUC) due to a decrease in drug clearance.[17] We suggest that a similar relationship between renal disease and mirtazapine CL exists in cats, based on the data presented here. There was a significant negative correlation between serum creatinine and clearance of mirtazapine ($r = - 0.69$ with $p = 0.0024$) when data from young normal cats, normal geriatric cats and CKD cats was analyzed using a Spearman rank correlation. Elimination half-life of mirtazapine is unaffected by degree of renal disease in humans. In this study, although a significant difference was not detected between the half-life in AMC and CKD cats, perhaps due to small sample size, there appears to be a notable difference between this data and that for normal cats. It is unknown to what degree non-linear pharmacokinetics may play a role in this observation, as it would be expected that half-life would become prolonged with increased exposure. As is found in humans,[10] differences in metabolism due to age may also play a role, as in this data set, pharmacokinetic parameters for the AMC group, particularly AUC, vary notably from those for young normal cats. Two cats from each IRIS stage were included in the present study, in order to represent the range of renal function that is encountered in clinical practice. This likely contributed to a greater standard deviation for some parameters, including half-life, and potentially affected the ability to

find a significant difference between groups. If cats from only one IRIS stage had been utilized, variability may have been reduced, and it may have been possible to demonstrate a difference between half-life in AMC and CKD cats.

In humans, sex is known to affect mirtazapine pharmacokinetics, with a shorter half-life and lower AUC in young males compared to females.[10] Thus an effort was made to control for this factor by having approximately equal numbers of each sex in each group. Unfortunately complete age and sex matching was not possible due to difficulty in recruiting CKD patients without concurrent illness, and this could have potentially have affected results. Study participant numbers were too small to determine if there was a significant effect of sex on metabolism of mirtazapine in cats.

In an effort to evaluate the duration of effect of the drug, assessments were made in the home environment during the pharmacodynamic study. Observations recorded by owners in a blinded fashion indicated that the majority of cats did experience clinical effects of the drug the night after the medication was administered and a statistically significant difference in appetite and behavior was noted for both HD and LD when compared to placebo. Although a few cats still experienced effects 24 hours post-administration, the majority of cats had no apparent clinical effect at that time point and no statistically significant difference in appetite or behavior was noted for either dose in comparison to placebo. These results conceivably may have been affected by unknown bias on the part of the owners or unknown factors affecting the cats in the 24 hours after their participation in the pharmacodynamic study.

A limitation of the pharmacokinetic study was the inability to determine true clearance as the drug was not given intravenously. There are several reasons why this was not performed. First of all, this study was performed in client-owned animals and the investigators were reluctant to administer a medication intravenously with no prior experience of this route, and no knowledge of possible adverse effects. In addition, as the pharmacodynamic study demonstrated a clinical effect of the oral dose used in the pharmacokinetic study, the authors did not believe the administering the medication by the IV route would provide clinically useful information. Thus the alternate measurement of clearance/bioavailability was used instead. This measurement operates under the assumption that bioavailability will not change

between groups. As a subjective non-statistically significant difference in $C_{max}$ was noted between groups, implying that absorption may have differed between the groups, there is a possibility that this assumption was not valid and results should be interpreted accordingly.

Another potential limitation of this study is the measurement of renal function by serum creatinine. The use of serum creatinine instead of glomerular filtration rate could have possibly affected the interpretation of the correlation between renal function and clearance of mirtazapine. Glomerular filtration rate is a more accurate assessment of renal function but is not practical for clinical assessment. Serum creatinine was used in this study in order to provide a clinically applicable tool on which to base a decision about mirtazapine administration, however as a result cats with subclinical kidney disease may have been inadvertently included in the AMC group. As muscle wasting is known to result in a lowering of serum creatinine,[18] cats selected for the study were of normal body condition and did not have marked muscle wasting.

The pharmacokinetic information gathered in this study was used to help determine appropriate dose intervals for cats with CKD in the subsequent clinical trial. Calculation of an accumulation factor for daily dosing compared to every other day dosing was performed. Although no evidence of drug accumulation was seen with the 48-hour dose interval (accumulation factor = 1.15), accumulation is potentially possible with daily dosing in CKD cats (accumulation factor = 1.57). This is in contrast to young normal cats, where no evidence of drug accumulation was found with daily dosing (accumulation factor = 1.2). However, as concentration may not reflect clinical effect, in order to fully understand the pharmacokinetic and pharmacodynamic implications of this dosing regimen in CKD cats, a clinical trial with repeated dosing every 48 hours was performed.

In the placebo controlled crossover clinical trial, repeated administration of 1.88 mg of mirtazapine every 48 hours for three weeks did indeed result in a statistically significant increase in appetite and subsequently weight. Additional benefits included a statistically significant increase in activity and a statistically significant decrease in vomiting. These results are consistent with descriptions of mirtazapine's anti-emetic and appetite stimulating properties for humans with renal disease and

anorexia associated with cancer chemotherapy.[19-21] No significant behavioral side effects were noted with the dosing regimen used in the clinical trial. However one cat did experience subclinically elevated ALT which subsequently resolved with discontinuation of mirtazapine. A similar case was reported in humans, where an individual experienced a dose-dependent asymptomatic elevation in liver enzymes that subsequently resolved after mirtazapine was discontinued.[22] The mechanism of this hepatopathy is unclear, but it is recommended that liver enzymes be monitored in patients receiving mirtazapine and that it not be used in patients that experience this side effect.

In conclusion, mirtazapine at a dose of 1.88 mg per cat is an effective appetite stimulant in both healthy young cats and cats with CKD. Pharmacokinetic and pharmacodynamic data lead us to conclude that daily dosing is appropriate in young normal cats, but as CKD appears to slow the clearance of mirtazapine, every other day dosing is recommended in cats with CKD. In cats with CKD additional benefits of weight gain and decreased vomiting were appreciated and this drug will be a useful adjunctive therapy in the management of anorexia associated with this common disease. Age and/or subclinical kidney disease may also potentially affect the metabolism of mirtazapine in cats. This information should be considered when clinicians are determining dosing regimens for their patients.

## 4.7 References


**1.** Roudebush P, Polzin DJ, Ross SJ, Towell TL, Adams LG, Dru Forrester S. Therapies for feline chronic kidney disease. What is the evidence? *J Feline Med Surg.* Mar 2009;11(3):195-210.

**2.** Polzin DJ. Chronic kidney disease in small animals. *Vet Clin North Am Small Anim Pract.* Jan 2011;41(1):15-30.

**3.** Sanderson BJ. Management of anorexia. In: Bonagura J, ed. *Current Veterinary Therapy XIII*. Philadelphia: WB Saunders, Co; 2000:69-74.

**4.** Kopple JD. Effect of nutrition on morbidity and mortality in maintenance dialysis patients. *Am J Kidney Dis.* Dec 1994;24(6):1002-1009.

**5.** Parker VJ, Freeman LM. Association between body condition and survival in dogs with acquired chronic kidney disease. *J Vet Intern Med.* Nov-Dec 2011;25(6):1306-1311.

**6.** Elliott J, Rawlings JM, Markwell PJ, Barber PJ. Survival of cats with naturally occurring chronic renal failure: effect of dietary management. *J Small Anim Pract.* Jun 2000;41(6):235-242.

**7.** Plantinga EA, Everts H, Kastelein AM, Beynen AC. Retrospective study of the survival of cats with acquired chronic renal insufficiency offered different commercial diets. *Vet Rec.* Aug 13 2005;157(7):185-187.

**8.** Ross SJ, Osborne CA, Kirk CA, Lowry SR, Koehler LA, Polzin DJ. Clinical evaluation of dietary modification for treatment of spontaneous chronic kidney disease in cats. *J Am Vet Med Assoc.* Sep 15 2006;229(6):949-957.

**9.** Plotnick A. Feline chronic renal failure: long-term medical management. *Compend Contin Educ Vet.* Jun 2007;29(6):342-344, 346-350; quiz 351.

**10.** Timmer CJ, Sitsen JM, Delbressine LP. Clinical pharmacokinetics of mirtazapine. *Clin Pharmacokinet.* Jun 2000;38(6):461-474.

**11.** Cahill C. Mirtazapine as an antiemetic. *Veterinary Forum.* 2006:34-36.

**12.** Lainesse C, Frank D, Meucci V, Intorre L, Soldani G, Doucet M. Pharmacokinetics of clomipramine and desmethylclomipramine after single-dose intravenous and oral administrations in cats. *J Vet Pharmacol Ther.* Aug 2006;29(4):271-278.

**13.** Doherty B, Rodriguez V, Leslie JC, McClean S, Smyth WF. An electrospray ionisation tandem mass spectrometric investigation of selected psychoactive pharmaceuticals and its application in drug and metabolite profiling by liquid chromatography/electrospray ionisation tandem mass spectrometry. *Rapid Commun Mass Spectrom.* 2007;21(13):2031-2038.

**14.** Wagner J. *Pharmacokinetics for the Pharmaceutical Scientist*. Lancaster, PA: Technomic Publishing Company, Inc.; 1993.

**15.** Panetta JC, Iacono LC, Adamson PC, Stewart CF. The importance of pharmacokinetic limited sampling models for childhood cancer drug development. *Clin Cancer Res.* Nov 1 2003;9(14):5068-5077.

**16.** Littel R. *SAS for mixed models*. 2nd ed. Cary, NC: SAS Institute Inc.; 2006.

**17.** Bengtsson F, Hoglund P, Timmer CJ. Mirtazapine oral single dose kinetics in patients with different degrees of renal failure. *Human Psychopharmacology.* 1998:357-365.

**18.** Baxmann AC, Ahmed MS, Marques NC, et al. Influence of muscle mass and physical activity on serum and urinary creatinine and serum cystatin C. *Clin J Am Soc Nephrol.* Mar 2008;3(2):348-354.

**19.** Kast RE, Foley KF. Cancer chemotherapy and cachexia: mirtazapine and olanzapine are 5-HT3 antagonists with good antinausea effects. *Eur J Cancer Care (Engl).* Jul 2007;16(4):351-354.

**20.** Pae CU. Low-dose mirtazapine may be successful treatment option for severe nausea and vomiting. *Prog Neuropsychopharmacol Biol Psychiatry.* Aug 30 2006;30(6):1143-1145.

**21.** Kim SW, Shin IS, Kim JM, et al. Effectiveness of mirtazapine for nausea and insomnia in cancer patients with depression. *Psychiatry Clin Neurosci.* Feb 2008;62(1):75-83.

**22.** Adetunji B, Basil B, Mathews M, Osinowo T. Mirtazapine-associated dose-dependent and asymptomatic elevation of hepatic enzymes. *Ann Pharmacother*. Feb 2007;41(2):359.

# CHAPTER 5: ASSESSMENT OF MESENCHYMAL STEM CELL THERAPY FOR PALLIATION OF FELINE CHRONIC KIDNEY DISEASE

I would like to acknowledge Dr. Tracy Webb for laboratory expertise, cell culture assistance and much advice that was critical to the success of this project. I would also like to acknowledge Lauren Habenicht for her work on urinary cytokines and her help with ELISAs.

## 5.1 Abstract

**Background:** Feline CKD is characterized by tubulointerstitial inflammation, fibrosis and progressive loss of renal function. Administration of MSC has been beneficial in rodent models, particularly with respect to reducing intra-renal inflammation and suppressing fibrosis.

**Aim:** The specific aims of these studies were to a) harvest, culture and characterize feline MSC, b) assess the effects of intrarenal administration of autologous MSC in CKD cats and c) assess the effects of intravenous administration of allogeneic cryopreserved MSC in CKD cats.

**Methods:** An initial pilot study was conducted in which autologous MSC were harvested from bone marrow or adipose tissue, expanded, and injected unilaterally into the renal cortex of 2 normal and 4 CKD cats. Complete blood count, serum biochemistry, urinalysis and glomerular filtration rate (GFR) via nuclear scintigraphy were monitored. A second pilot study was conducted where adipose-derived allogeneic cryopreserved MSC were intravenously administered to CKD cats in two different dose groups every two weeks for three treatments. Complete blood count, serum biochemistry, urinalysis, urine protein: creatinine ratio, GFR by iohexol clearance, and urinary cytokines were monitored.

**Results:** MSC were successfully harvested and cultured from bone marrow and adipose sources; the latter was preferred for ease of collection, expansion and superior yield. Intrarenal injection did not induce immediate or delayed adverse effects. Two CKD cats that received adipose-derived MSC via intrarenal

injection experienced modest improvement in GFR and a small decrease in serum creatinine concentration. In the intravenous study, six cats received 2 x $10^6$ MSC per injection (Group A) and experienced a significant decrease in serum creatinine with negligible side effects. Five cats received 4 x $10^6$ MSC IV per injection (Group B) and side effects included vomiting during infusion and increased respiratory rate. Variable decreases in serum creatinine, increases in GFR by iohexol clearance, and changes in urinary cytokines were seen in these cats.

**Conclusions:** Feline MSC can be obtained from bone marrow or adipose tissue, expanded and cryopreserved for therapeutic use. Intrarenal MSC injection may result in mild improvement in renal function, but the number of sedations and interventions required would likely preclude widespread clinical application. Efficacy of repeated intravenous injection of cryopreserved MSC appears variable and may result in an increased incidence of side effects at higher doses.

## 5.2 Introduction

CKD is a common condition in the geriatric cat and is characterized by tubulointerstitial damage, fibrosis and progressive loss of renal function. Currently there is no definitive therapy short of renal transplantation, and therefore novel therapeutic options are highly desirable. Recently the use of MSC for the treatment of CKD has been a focus of research interest. Mesenchymal stem cells modulate immune responses, are very non-immunogenic and can be transferred to fully allogeneic recipients and still retain their immunologic properties.[2,3] The immunological properties of MSC include inhibition of lymphocyte proliferation and cytokine production, suppression of dendritic cell function and alteration of dendritic cell cytokine production, and suppression of IFN-g production by NK cells.[3] As the underlying pathology of CKD is inflammation, the immunomodulatory properties of MSC can potentially be applied therapeutically. The effects of MSC therapy have been investigated in several rodent CKD models including genetic disease, glomerulonephritis and experimentally-induced CKD.[4-10] In the majority of experimentally-induced CKD models investigated, MSC administration has resulted in beneficial

changes, as evidenced by improvement in renal functional parameters, decreased intra-renal inflammation and reduction of renal fibrosis.[11-15]

The ideal route for MSC administration in renal disease has also been a focus of research. In previous CKD rodent model studies MSC have been administered systemically by the IV route, directly into the renal artery, directly into the renal parenchyma, and into the renal subcapsular space.[11-15] Recently, systemic IV administration has become more widely used due to the prevailing theory that MSC mechanism of action is predominantly paracrine in nature. Although several studies have demonstrated incorporation of small numbers of MSC into the renal parenchyma, and it has been proposed that some of these MSC may actually differentiate into functional renal tubular epithelial cells, this theory remains controversial.[16-18] Other investigators propose that paracrine effects from the injected MSC are more important than the effects of direct cellular incorporation into the kidney.[19,20]

The purpose of the pilot studies described in this chapter was to assess the feasibility of MSC transfer in normal cats and in cats with CKD. In this body of work we have assessed two routes of administration of MSC: directly into the kidney, and intravenous. Unilateral intrarenal transfer of MSC was chosen to allow internal comparison between the injected kidney and the non-injected kidney using GFR as determined by nuclear scintigraphy. Intravenous injection was chosen to assess the effects of systemic administration, which is technically less challenging and more feasible in a clinical setting. These studies were designed to test the hypotheses that MSC could be safely administered to cats with CKD by multiple routes and that MSC injection would result in improvement in kidney function.

## 5.3 Intra-renal Autologous MSC Pilot Study

### 5.3.1 Materials and Methods

#### *5.3.1a Healthy Specific Pathogen Free (SPF) Cats.*

Two healthy SPF purpose-bred domestic shorthaired cats (Andrea D. Lauerman SPF Cat Colony Resource, Fort Collins, CO) were utilized for the study. Both cats were 1.5 years of age (one castrated male, one intact female). The cats were adopted into private homes at the conclusion of the study. The study was approved by the Colorado State University Institutional Animal Care and Use Committee.

#### *5.3.1b Study Population of Cats with Stable CKD.*

Cats with stable CKD were recruited from the patient population at the Veterinary Teaching Hospital at Colorado State University. Cats were determined to have stable CKD based on two repeated biochemical evaluations performed at least two weeks apart. Pretreatment evaluation included complete blood count (CBC), biochemistry profile, urinalysis, urine culture, blood pressure, total T4, urine protein creatinine ratio (UPC), FeLV/FIV serology, abdominal radiographs, and a renal ultrasound. Cats were excluded from the study if they had evidence of ureteroliths, pyelonephritis, uncontrolled hypertension, or concurrent systemic disease. Administration of concurrent supportive therapies was allowed provided there were no changes in therapy during the study period. The study was approved by the Institutional Animal Care and Use Committee at Colorado State University, and all owners reviewed and signed consent forms prior to participation in the study.

#### *5.3.1c Autologous MSC Collection and Culture*

For collection of bone marrow or adipose tissue biopsies, cats were sedated with ketamine (Fort Dodge, Fort Dodge, IA) 3.3-4.8 mg/kg, IV, once (dose repeated once if needed), midazolam (Baxter HealthCare Corp., Deerfield, IL) 0.1 mg/kg, IV, once, and butorphanol (Fort Dodge, Fort Dodge, IA) 0.1 mg/kg IV, once. Intravenous fluids were administered during sedation at 5ml/kg/hr and blood pressure, pulse and respiration were monitored. After sterile preparation of the skin and a local injection of

lidocaine, approximately 1 ml of bone marrow was collected from the proximal humerus and placed into plastic tissue culture flasks (BD Biosciences, San Jose, CA) in MSC medium (low-glucose DMEM, 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine, 1% essential amino acids without L-glutamine, 1% nonessential amino acids, 1% bicarbonate Invitrogen/Gibco, Carlsbad, CA) plus 15% fetal bovine serum (Cell Generation, Fort Collins, CO). The non-adherent cells were washed off at 48 hours, the remaining bmMSC were incubated until approximately 70% confluent with media changes every 2-3 days. The cells were harvested with trypsin (Invitrogen/Gibco, Carlsbad, CA) and passaged until adequate cell numbers were obtained for injection.

Adipose tissue was obtained from a subcutaneous site on the ventral abdomen just caudal to the umbilicus. For preparation of the adipose tissue for culture, the tissue was minced and digested with 1mg/ml collagenase (Sigma Aldrich, St. Louis, MO) for 30 minutes at 37°C. The sample was centrifuged and the stromal vascular fraction was washed, plated in MSC medium and expanded as described above for bmMSC.

***5.3.1d Characterization of feline MSC***

Adipose-derived MSC (aMSC) and bone marrow-derived MSC (bmMSC) were characterized by cell surface marker expression using flow cytometry and a panel of feline-specific and cross-reactive antibodies specific for surface determinants expressed by MSC from other species.[21-23] Specifically, feline MSC were analyzed for surface expression of CD44 ( antibody clone:IM7, eBioscience, San Diego, CA), CD105 (antibody clone:SN6, eBioscience, San Diego, CA), and CD90 (antibody clone:eBio5E10, eBioscience, San Diego, CA ). MSC were also assessed for expression of CD4 (anti-feline antibody clone:3-4F4, Southern Biotech, Birmingham, AL) and MHC class II (antibody clone: TÜ39, BD Biosciences, San Jose, CA). Samples were analyzed using a Cyan ADP flow cytometer (Beckman Coulter, Brea, CA).

*In vitro* differentiation assays were conducted to confirm the multipotency of feline MSC, as assessed by their ability to differentiate into three cell lineages (osteoblasts, chondrocytes, and adipocytes) that are characteristic of MSC.[24] Assays were performed by incubating confluent MSC with medium supplemented with factors to stimulate differentiation.[24] At the end of the differentiation period, cells were fixed with 10% neutral buffered formalin and stained with Oil red O (Sigma Aldrich, St. Louis, MO) for presence of lipid, with toluidine blue (Richard-Allan Scientific, Kalamazoo, MI) for cartilage matrix, or with Alizarin red (Sigma Aldrich, St. Louis, MO) for the presence of calcium.[24] MSC cultured in MSC media alone under identical conditions were used as differentiation controls.

### *5.3.1e Intrarenal Injection of MSC*

Increasing doses of autologous MSC were administered to the enrolled cats. Each recipient cat was sedated using IV administration of ketamine 3.3-4.8 mg/kg, once (dose repeated once if needed), midazolam 0.1 mg/kg, and butorphanol 0.1 mg/kg,placed in lateral recumbency, and monitored as previously described. Harvested MSC were suspended in PBS, divided into three 150-200ul aliquots and injected using a 25 gauge needle into three sites in the renal cortex (side was randomly determined) under ultrasound guidance. Cats enrolled in the study were observed for adverse effects immediately following intrarenal MSC injection, including physical examination to assess pulse, respiration, mucus membrane color and abdominal or renal discomfort. Cortical injection sites were assessed for hemorrhage 1 hour and 24 hours after injection using ultrasonography.

### *5.3.1f Determination of GFR by Nuclear Scintigraphy*

GFR was assessed in each kidney of cats injected with MSC using nuclear scintigraphy and was performed just prior to injection of MSC, 7 days after injection, and 30 days after injection. Cats were sedated (ketamine 3.3-4.8 mg/kg and butorphanol 0.1mg/kg IV once) at a standard time before the procedure. The same technician performed all of the procedures for each individual cat. For each procedure 1.0 mCi of Tc99m labeled DTPA (Cardinal Health, Dublin, OH) was injected intravenously via

a catheter placed in a standard location in each cat. Images were obtained using GE Millenium SPECT system (GE Healthcare, Waukesha, WI). Three independent radiologists evaluated the GFR data, and a mean GFR value for each kidney as well as a global value was determined. To assess the degree of intra-patient variability in repeated GFR measurements as determined by nuclear scintigraphy, three cats with CKD that did not receive MSC underwent nuclear scintigraphy GFR assessments on two occasions, one week apart. Data from these cats was used to provide information on inherent GFR variability and help interpret the results of GFR measurements made in cats with CKD treated with MSC.

#### *5.3.1g Clinical Monitoring of Treated Cats*

Each treated cat underwent physical examination and had routine blood work (CBC, serum biochemistry, urinalysis, and determination of UPC) performed immediately prior to MSC injection and on day 7, day 30, and day 60 after MSC injection.

#### *5.3.1h Histopathology*

Two study cats with Stage IV CKD were humanely euthanized in consultation with their owners due to progressive renal failure. The tissues of both the MSC-injected and non-injected kidneys were examined histopathologically using hematoxylin and eosin stained sections.

#### *5.3.1i Statistical Analysis*

Changes in GFR, serum creatinine, BUN and PCV data over time in the MSC-injected cats were evaluated by repeated measures ANOVA, followed by Bonferroni's correction. Values were considered statistically different if $p < 0.05$. Statistical analyses were done using Prism5 software (GraphPad, San Diego, CA).

## 5.3.2 Results

### *5.3.2a Cats*

Nine cats (2 healthy cats, 7 cats with CKD) were enrolled in the study. Two healthy cats and four CKD-affected cats were ultimately treated with MSC in the study. Three additional cats were enrolled for GFR variability studies. **Table 5.1** summarizes the treatments received for all cats. Of the seven CKD cats were enrolled in the study initially, bmMSC could not be cultured to produce sufficient treatment quantity in three of the cats; 1 cat received bmMSC, and 3 cats received aMSC.

**Table 5.1.** Summary of demographics of cats participating in the intra-renal MSC pilot study.

**Table 1.** Description of cats included in the study.

| Cat | Group | Signalment | IRIS stage and creatinine (mg/dl) | Treatment |
|---|---|---|---|---|
| 1 | Young healthy | 1.5-year MC DSH | IRIS: N/A<br>Creatinine: 1.7<br>UPC: 0.1 | $1 \times 10^5$ bmMSC injected into the right kidney |
| 2 | Young healthy | 1.5-year FI DSH | IRIS: N/A<br>Creatinine: 1.3<br>UPC: 0.1 | $1 \times 10^5$ bmMSC injected into the left kidney |
| 3 | CKD + bmMSC | 6-year-old MC DLH | IRIS: IV<br>Creatinine: 5.4<br>UPC: 1.7 | $1 \times 10^5$ bmMSC injected into the left kidney |
| 4 | CKD + bmMSC | 15-year MC Tonkinese | IRIS: III<br>Creatinine: 4.3<br>UPC: 0.3 | Unable to expand sufficient bmMSC for therapy |
| 5 | CKD + bmMSC | 17-year MC Siamese | IRIS: II<br>Creatinine: 2.6<br>UPC: not performed | Unable to expand sufficient bmMSC for therapy |
| 6 | CKD + bmMSC | 14-year MC DSH | IRIS: II<br>Creatinine: 2.0<br>UPC: 0.2 | Unable to expand sufficient bmMSC for therapy |
| 7 | CKD + aMSC | 9-year FS Siamese | IRIS: III<br>Creatinine: 3.5<br>UPC: 0.1 | $1 \times 10^6$ aMSC injected into the left kidney |
| 8 | CKD + aMSC | 9-year MC DSH | IRIS: II<br>Creatinine: 2.6<br>UPC: 0.2 | $2 \times 10^6$ aMSC injected into the left kidney |
| 9 | CKD + aMSC | 7-year MC DSH | IRIS: IV<br>Creatinine: 6.5<br>UPC: 0.3 | $4 \times 10^6$ aMSC injected into the left kidney |
| 10 | CKD no MSC | 9-year MC DSH | IRIS: II<br>Creatinine: 2.3<br>UPC: 0.3 | GFR repeatability only |
| 11 | CKD no MSC | 12-year FS DSH | IRIS: III<br>Creatinine: 3.3<br>UPC: 0.1 | GFR repeatability only |
| 12 | CKD no MSC | 13-year MC DSH | IRIS: II<br>Creatinine: 2.6<br>UPC: 0.2 | GFR repeatability only |

DSH = domestic shorthair, FS = female spayed, MC = male castrated; IRIS = Internation Renal Interest Society.

Feline bmMSC and aMSC developed over a 1-2 week period into a relatively homogeneous population of plastic-adherent cells with fibroblast-like morphology (**Figure 5.1**). For three cats that were initially enrolled in the study, bmMSC failed to expand to sufficient quantities for injection. In contrast, aMSC and were easier to culture and expand, and sufficient cells for treatment were easily obtained from all three CKD cats enrolled in this part of the study.

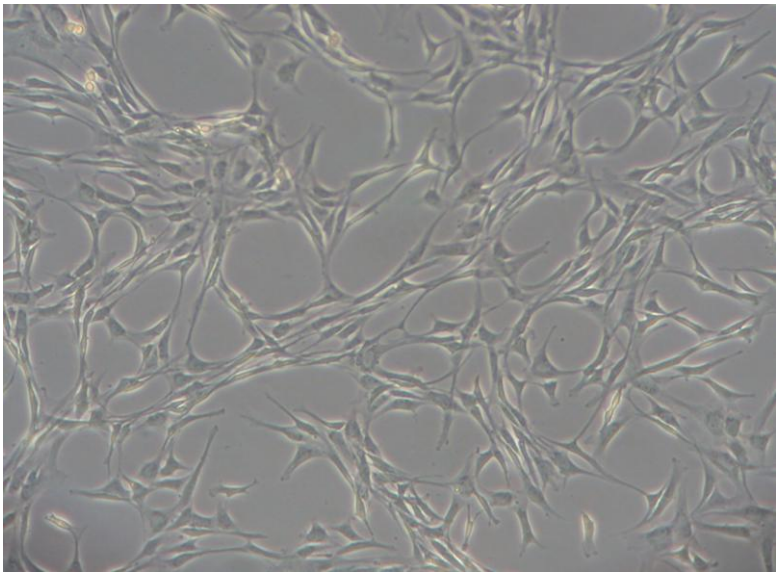


**Figure 5.1:** Phenotype of feline adipose tissue-derived MSC in culture. Feline adipose derived MSC were established from adipose tissue biopsies, as described in Materials and Methods. Cells assumed a typical elongated morphology, as described previously for feline MSC.[1] (Feline adipose MSC, magnification 10x).

### *5.3.2c Characterization of Feline MSC.*

Both bmMSC and aMSC expressed high levels of CD44, CD90 and CD105 and had extremely low levels of expression of CD4 and MHC class II (**Figure 5.2**), which was consistent with the phenotype described previously for feline MSC.[1] Both bmMSC and aMSC were capable of tri-lineage differentiation (**Figure 5.3**).

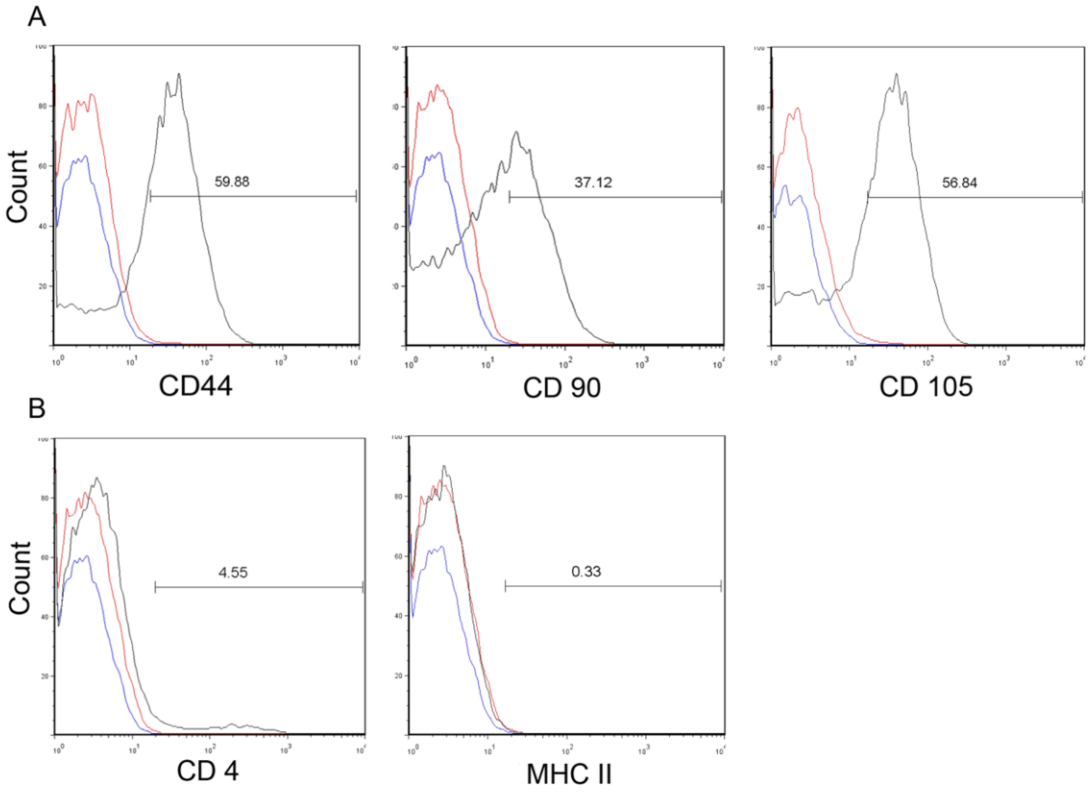



**Figure 5.2:** Expression of cell surface markers by feline adipose-derived MSC as determined by flow cytometry. Feline adipose MSC expressed high surface levels of CD44, CD90, and CD105 (panel A), but did not express CD4 or MHC class II (panel B). Similar results were obtained with adipose MSC from 4 additional cats.

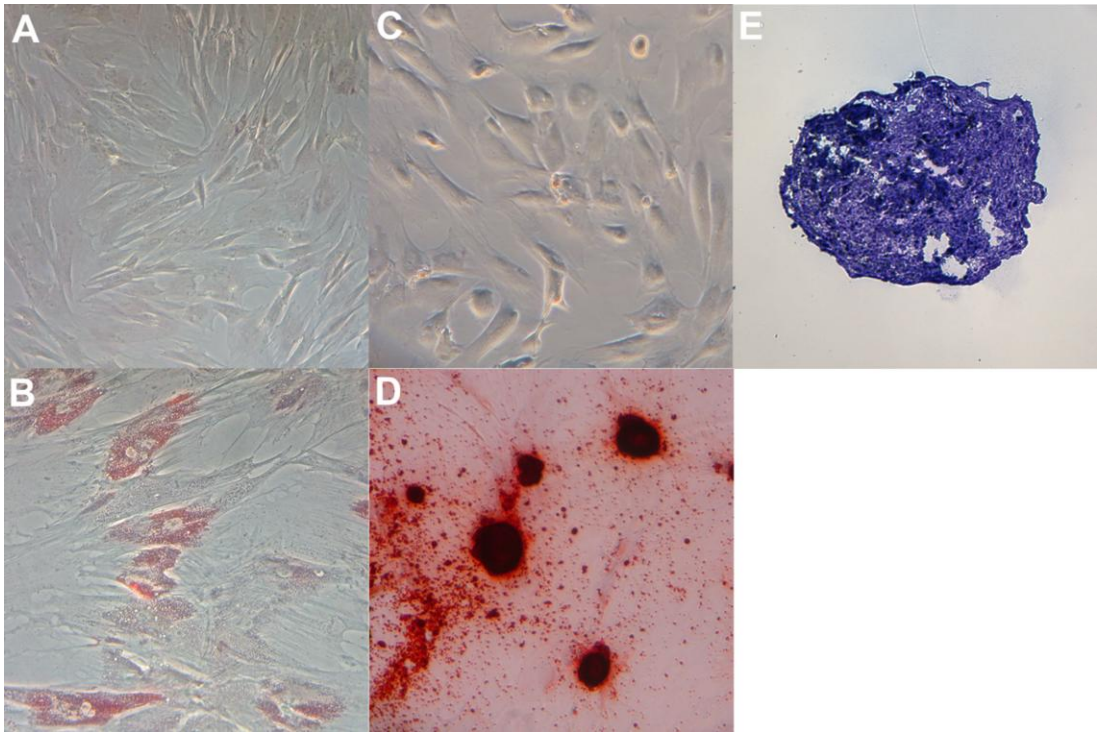



**Figure 5.3:** Trilineage differentiation of feline aMSC. A). Control aMSC incubated in standard media stained with oil red O. B). aMSC produced intracellular lipid vacuoles when incubated in adipocytic differentiation media for 21 days. C). Control aMSC incubated in standard media stained with alizarin red. D.) aMSC stained positive for calcium with alizarin red following differentiation into osteocytic phenotype after 21 days of incubation in differentiation media. E) Cryosection of pellets of cartilage matrix (stained with toluidine blue) formed by aMSC when exposed to chondrocytic differentiation media for 21 days.

### *5.3.2d Short-term Safety of Intrarenal Injection of MSC in Cats*

Hemorrhage was not observed by ultrasound examination 1 hour or 24 hours post-injection in any of the treated cats. The cats appeared clinically normal, and renal discomfort was not elicited on abdominal palpation. One of the healthy young cats developed transient, microscopic hematuria 24 hours after MSC injection, but was not otherwise clinically affected.

### *5.3.2e Assessment of GFR Variability*

GFR values were determined on two separate occasions for three cats with CKD that did not receive MSC injection. These studies revealed that the mean total GFR for the three untreated cats was 1.46 ± 0.28 ml/kg/min on week 1 and 1.55 ± 0.08 ml/kg/min on week 2. The mean calculated variation between repeated GFR values for the three untreated cats with CKD between week 1 and week 2 was 9.6% (**Figure 5.4**).

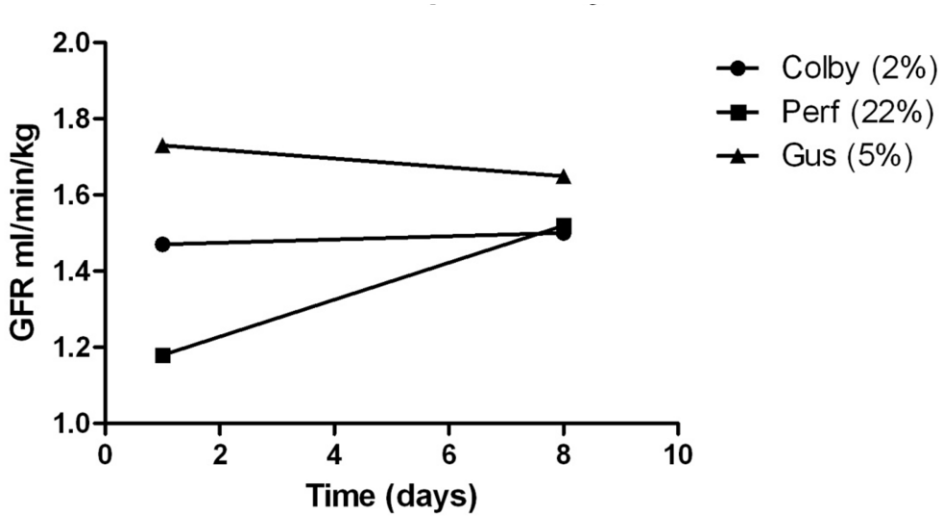



**Figure 5.4:** Assessment of repeatability with GFR by nuclear scintigraphy. Three cats with CKD that were not treated with MSC had GFR studies performed twice, one week apart. Mean calculated variation was 9.6%.

### *5.3.2f Effects of MSC Injection on GFR*

In the two healthy, young cats that received intrarenal MSC injection, the mean pre-treatment global GFR (sum of both kidneys) as determined by nuclear scintigraphy was 3.3 ± 0.57 ml/kg/min (mean and SD), the mean 7 day GFR was 2.7 ± 0.86 ml.kg/min, and the mean 30 day GFR was 3.6 ± 1.5 ml/kg/min.

Renal function in the three cats with CKD that received unilateral intra-renal injection of MSC (one bmMSC, two aMSC) was evaluated immediately prior to MSC injection, on day 7 post-injection, and on day 30 post-injection. A fourth cat was unable to undergo GFR monitoring via nuclear scintigraphy due to fractious nature. The mean pre-treatment global GFR for the three cats with CKD was 0.88 ± 0.53 ml/kg/min, (median 0.72 ml/kg/min) which was 35% of the reported normal global GFR value for healthy adult cats (2.5ml/kg/min).[27] Following MSC transfer, mean global GFR value for the 3 MSC-injected cats was 1.1 ± 0.59 ml/kg/min (median 0.74 ml/kg/min) at 30 days, which though numerically increased was not significantly different from the pre-treatment global GFR value. Although the overall GFR in treated cats was not significantly altered by MSC injection, CKD cats 7 and 8 had global GFR increases of 16% and 55% from baseline respectively as compared to the 9.6% GFR variability determined in non-treated cats. In cat 3, who received bmMSC, no change from baseline was observed.

GFR values were also determined for individual kidneys of cats with CKD injected with MSC (**Figure 5.5**). In the MSC-injected kidney of all three treated cats, the mean pre-treatment GFR value was 0.45 ± 0.39 ml/kg/min, (median 0.26 ml/kg/min) compared with a mean value of 0.58 ± 0.54 ml/kg/min (median 0.28 ml/kg/min) determined on day 30. For the non-injected kidney, the mean pre-treatment value was 0.42 ± 0.14 ml/kg/min, (median 0.46 ml/kg/min) compared to 0.47 ± 0.05 ml/kg/min (median 0.46 ml/kg/min) on day 30. These data suggested a trend towards increasing GFR values for the injected kidney.

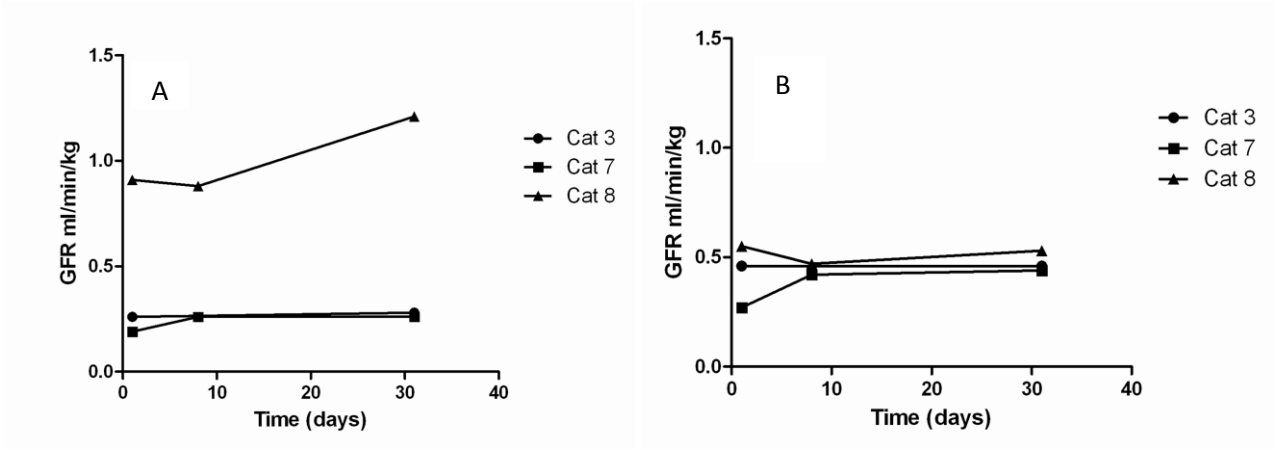



**Figure 5.5:** Changes in GFR over time in cats with CKD that received unilateral intrarenal MSC injections. GFR via nuclear scintigraphy was evaluated prior to treatment, on day 7 and day 30 in 3 cats for the injected kidney (A) and non-injected kidney (B).

***5.3.2g Effects of MSC Injection on Clinical Parameters of Renal Function and Outcome.***

In the two healthy cats that received intra-renal injections with bmMSC, no significant changes in relevant laboratory values (serum creatinine, BUN, PCV, UPC) measured before and after MSC injection (data not shown) were observed.

In CKD cats 7 and 8, which received aMSC, a modest though statistically insignificant improvement in serum creatinine concentration was noted, particularly at the 60 day time point (**Figure 5.6**). The PCV, BUN, and UPC values were unchanged following treatment. Cat 7 was euthanized for acute lymphocytic leukemia four months after completing the MSC study, an outcome which was considered unrelated to the study itself. Cat 8 was still alive with stable CKD 10 months after MSC injection.

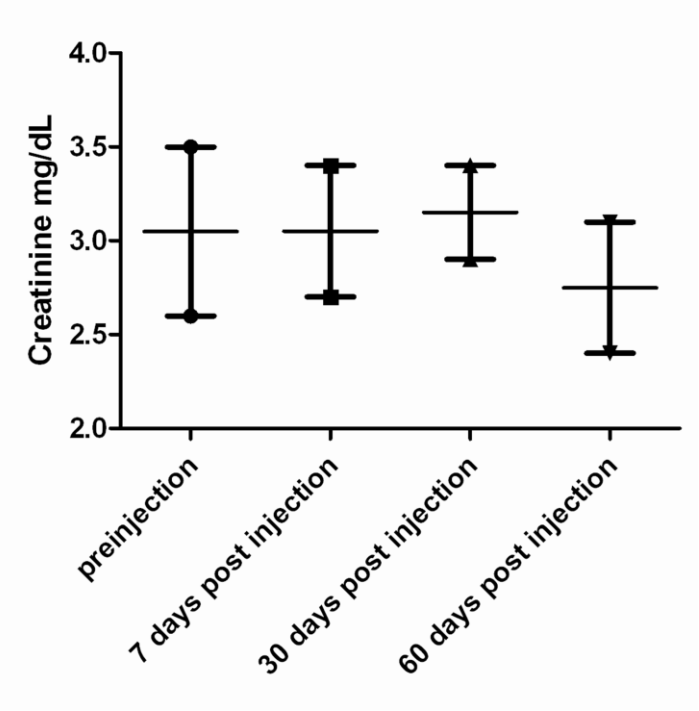



**Figure 5.6:** Changes in serum creatinine concentration over time in cats with CKD that received unilateral, intrarenal MSC injections. Serum creatinine concentrations were evaluated prior to treatment, on day 7, day 30 and on day 60 in 2 cats that completed a 60-day trial of MSC injection therapy.

In CKD cat 3 (Stage IV: creatinine 5.4 mg/dL) that received bmMSC, a small improvement in serum creatinine was observed on day 7 (creatinine 5.1 mg/dL) and day 30 (creatinine 5.2 mg/dL), and the urine protein-creatinine ratio (UPC) value increased over the same time period (initial UPC 1.1; 30 day UPC 3.3). This cat traveled some distance for its 60 day recheck (800 miles) and although the owners perceived the cat was doing well at home before travel, renal function in this cat had declined substantially upon arrival (creatinine 8.2 mg/dL), and the cat was subsequently euthanized at day 100.

In CKD cat 9 (Stage IV: creatinine 6.5 mg/dL) that received aMSC, a small change in creatinine was seen at 7 day (creatinine 6.1 mg/dL) but by day 30 this cat was clinically less stable (creatinine 8.4) and was subsequently euthanized at day 42.

### *5.3.2h Histopathology*

Histopathologic examination of the kidneys was performed in both CKD cats 3 and 9. In cat 3, there were abundant numbers of lymphocytes and plasma cells found multifocally, expanding the interstitium as well as extensive interstitial fibrosis. Tubules were frequently lost, atrophied and ectatic with some evidence of regeneration and frequently contained luminal protein casts and scattered calcium oxalate crystals. Tubule mineralization was seen within the cortex and medulla. Periglomerular fibrosis, glomerulosclerosis, mineralization and atrophy were present in 50% of glomeruli. In cat 9 there were lymphocytes, plasma cells and fewer neutrophils multifocally throughout the interstitium. Tubular loss, atrophy and regeneration were noted, often associated with fibrosis. Numerous tubules contained sloughed epithelial cells, as well as cellular, protein and waxy casts. Multifocally, tubules contained intraluminal crystals that were crescent or circular with radiating spokes and polarized. There were crystals present within the pelvis, which was lined by hyperplastic epithelium. Periglomerular fibrosis, glomerulosclerosis and a thickened basement membrane were present in 95% of glomeruli. Examination of the MSC-injected kidney of both cats did not show evidence of pathological changes (including vascular anomalies, tissue disruption, or abnormal cell types) at the presumed injection sites.

## 5.4 Intravenous Allogeneic Cryopreserved MSC Pilot Study

### 5.4.1 Materials and Methods

#### *5.4.1a Cats*

Cats with stable CKD were recruited from the patient population at the Veterinary Teaching Hospital at Colorado State University. Cats were determined to have stable CKD based on two repeated biochemical evaluations performed at least two weeks apart. Pretreatment evaluation included complete blood count (CBC), biochemistry profile, urinalysis, urine culture, blood pressure, total T4, urine protein creatinine ratio (UPC), FeLV/FIV serology, abdominal radiographs, and a renal ultrasound. Cats were excluded from the study if they had evidence of ureteroliths, pyelonephritis, uncontrolled hypertension, or concurrent systemic disease. Administration of concurrent supportive therapies was allowed provided there were no changes in therapy during the study period. The study was approved by the Institutional Animal Care and Use Committee at Colorado State University, and all owners reviewed and signed consent forms prior to participation in the study.

#### *5.4.1b Cell Preparation and Administration*

MSC were isolated from feline adipose tissue of donor SPF cats and expanded in culture as previously described (5.3.1c). After reaching passage 3, cells were harvested, divided into treatment aliquots, cryopreserved in freezing medium (11% DMSO, 45% MSC medium, 45% FBS) and stored in liquid nitrogen for no greater than one year prior to use. For injection cells were removed from liquid nitrogen, placed in MSC medium for 10 minutes, washed twice, counted and checked for viability. For the low dose aMSC group (Group A), $2 \times 10^6$ cells were resuspended in 5 ml Hank's balanced salt solution (HBSS) with 200 IU heparin sulfate and administered IV slowly by hand over 15 minutes: aMSC were administered at weeks 0, 2 and 4. For the high dose group (Group B), $4 \times 10^6$ cells were initially resuspended in 10 ml HBSS with 200 IU heparin sulfate and administered as a slow IV infusion over 30 minutes. However, the first cat to receive aMSC in this manner experienced an increased respiratory rate. Subsequently cells for the high dose group were resuspended in 20 ml HBSS/200 IU heparin and

administered over 45-60 minutes using a syringe pump with frequent agitation of the syringe to prevent settling of the cells. Cats in the high dose group received aMSC at weeks 2, 4, and 6. All three injections received by a treated cat were from the same donor cat. Cells from a total of 4 donor cats were used over the course of the study.

#### *5.4.1c Clinical Monitoring*

For Group A, each treated cat underwent physical examination, weighing and routine blood work consisting of CBC, serum biochemistry, urinalysis and urine protein:creatinine ratio (UPC) immediately prior to MSC injection at week 0, and at week 2,4, 6 and 8. For Group B, each treated cat underwent physical examination, weighing and routine blood work consisting of CBC, serum biochemistry and urinalysis at weeks 0, 2,4, 6 and 8. Additionally, each cat in Group B had a UPC and GFR study performed via iohexol clearance at weeks 0 and 8.

#### *5.4.1d GFR by Iohexol Clearance*

GFR by iohexol clearance has been described as a clinically applicable alternative to GFR by nuclear scintigraphy.[25] For this method, 300 mg/kg iohexol (Omnipaque, GE Health Incorporated, Princeton, NJ) is administered IV and blood samples are collected at 2, 3, and 4 hours after administration. Analysis is commercially available at the Michigan State University Diagnostic Center for Population and Animal Health. Assessment of GFR variability was performed by enrolling three CKD cats that did not receive MSC therapy, but underwent GFR by iohexol clearance at 0 and 8 weeks.

#### *5.4.1e Urinary Cytokine Analysis*

Urine samples were analyzed using commercially-available antigen-capture ELISA kits for feline IL-6, IL-8, and IL-10, canine MCP-1, canine VEGF, murine TGF-β1 (R&D Systems, Inc., Minneapolis, MN), and multispecies TGF-β1 (Invitrogen, Camarillo, CA). VEGF and TGF-β1 ELISAs have previously

been utilized in feline urine.[26,27] Due to the high level of protein homology between feline and canine MCP-1 (89% homology at the nucleic acid level, http://blast.ncbi.nlm.nih.gov/Blast.cgi).) we proposed that feline MCP-1 could be detected using a canine MCP-1 specific ELISA. Recombinant feline MCP-1 was not available to validate this assay for cats.

Urine samples were collected by cystocentesis for urinalysis, and an aliquot of urine was immediately frozen at -20ºC and then stored at -80ºC. Samples were thawed immediately prior to analysis and kept on ice during sample preparation. All samples were centrifuged at 2000 rpm (350 xg) for 5 minutes at 4 degrees C, and the supernatant was collected for analysis. ELISAs were run according to manufacturer instructions. Urine and serum creatinine concentrations were measured with a Roche Cobas Integra Chemistry Analyzer (Roche Diagnostics Limited, West Sussex, UK) at the Colorado State University Diagnostic Laboratory. All reported cytokine concentrations were normalized for differences in urine concentration as a urine cytokine-to-urine-creatinine-ratio. In order to maximize cytokine yield in the urine samples, additional sample processing steps were performed prior to running the IL-8, MCP-1, and VEGF ELISAs. For IL-8 detection, the urine supernatant was concentrated approximately 3-fold using a centrifuge filter system (Amicon-Ultra 3kDa, Millipore Corporation, Billerica, MA). Volumes before and after concentration were measured to calculate the concentration factor for each sample. Filtrates were then diluted 1 to 9 in 1% BSA prior to analysis by ELISA. For MCP-1 detection, urine supernatant was diluted 1 to 9 in 1% BSA, and for VEGF detection, urine supernatant was diluted 1 to 4 in 1% BSA.

### 5.4.2 Results

#### *5.4.2a Cats*

A total of 14 cats were involved in these studies. **Table 5.2** summarizes their participation.

**Table 5.2:** Summary of demographics of cats participating in the intravenous allogeneic cryopreserved studies.

| Cat | Group | Signalment | IRIS stage and creatinine (mg/dL) | Treatment |
|---|---|---|---|---|
| **1** | CKD<br>Low dose | 10 yr MC DSH | IRIS: II<br>Creatinine: 2.5<br>UPC: 0.2 | 2 x $10^6$ MSC IV x 3 treatments |
| **2** | CKD<br>Low dose | 15 yr FS DSH | IRIS: III<br>Creatinine: 3.5<br>UPC: 0.1 | 2 x $10^6$ MSC IV x 3 treatments |
| **3** | CKD<br>Low dose | 7 yr MC Siamese | IRIS: III<br>Creatinine: 4.3<br>UPC: 0.2 | 2 x $10^6$ MSC IV x 3 treatments |
| **4** | CKD<br>Low dose | 12 yr MC DLH | IRIS: II<br>Creatinine: 2.4<br>UPC: 0.2 | 2 x $10^6$ MSC IV x 3 treatments |
| **5** | CKD<br>Low dose | 15 yr MC DSH | IRIS: II<br>Creatinine: 2.3<br>UPC: 0.1 | 2 x $10^6$ MSC IV x 3 treatments |
| **6** | CKD<br>Low dose | 15 yr FS Siamese | IRIS: III<br>Creatinine: 3.5<br>UPC: 0.2 | 2 x $10^6$ MSC IV x 3 treatments |
| **7** | CKD<br>High dose | 11 yr MC DSH | IRIS: II<br>Creatinine: 1.9<br>UPC: 0.1 | 4 x $10^6$ MSC IV x 3 treatments |
| **8** | CKD<br>High dose | 11 yr FS DSH | IRIS: II<br>Creatinine: 2.6<br>UPC: 0.1 | 4 x $10^6$ MSC IV x 3 treatments |
| **9** | CKD<br>High dose | 18 yr FS DSH | IRIS: II<br>Creatinine: 2.8<br>UPC: 0.2 | 4 x $10^6$ MSC IV x 3 treatments |
| **10** | CKD<br>High dose | 15 yr MC DSH | IRIS: II<br>Creatinine: 2.2<br>UPC: 0.1 | 4 x $10^6$ MSC IV x 3 treatments |
| **11** | CKD<br>High dose | 7 yr MC DSH | IRIS: II<br>Creatinine: 3.7<br>UPC: 0.1 | 4 x $10^6$ MSC IV x 3 treatments |
| **12** | CKD no MSC | 15 yr MC DSH | IRIS: II<br>Creatinine: 2.3 | GFR repeatability only |
| **13** | CKD no MSC | 16 yr MC DSH | IRIS: II<br>Creatinine: 2.1 | GFR repeatability only |
| **14** | CKD no MSC | 16 yr MC DSH | IRIS: II<br>Creatinine: 1.8 | GFR repeatability only |

### *5.4.2b Short-term Safety of Intravenous Allogeneic MSC Administration*

Group A cats received 3 doses of MSC without apparent adverse effects, appearing clinically normal and undisturbed. However, Group B cats experienced transient adverse effects including vomiting (2 cats) and increased respiratory rate (4 cats). Vomiting and nausea occurred almost immediately after initiating the first injection. Administration of diphenhydramine 1 mg/kg subcutaneously (SQ) and maropitant 1mg/kg SQ resulted in cessation of clinical signs. Premedication with diphenhydramine and maropitant at subsequent injections prevented any adverse reaction. Increased respiratory rate was subtle in all cats but one. Increased respiratory rate generally occurred after approximately two-thirds of the aMSC had been administered. One cat however, had mildly increased respiratory rate at the first injection, a normal second injection, and overt respiratory distress at the time of the third injection after approximately one-third of the cells had been administered. This was characterized by a sudden onset of increased respiratory rate that quickly lead to nasal flare, open mouth breathing and an orthopnic stance with head extended and elbows out. The injection was aborted, the cat was moved to critical care and placed in an oxygen chamber and diphenhydramine was administered. After 20 minutes no improvement was noted, and the cat became notably nauseated (foaming at the mouth and subsequently vomiting). Maropitant 1 mg/kg SQ and dexamethasone 0.05 mg/kg IV were administered. Clinical signs began to abate within 20 minutes. Open mouth breathing and orthopnea improved within 1 hour. Increased respiratory rate continued for the next 8 hours, but by 24 hours post injection the cat was clinically normal and was discharged without further incident.

### *5.4.2c Effects of MSC Administration on Clinical Parameters*

Serum creatinine concentrations decreased significantly (p=0.01) in group A cats, but were unchanged in Group B cats (**Figures 5.7 and 5.8**). One cat was removed from analysis of serum creatinine because the owners failed to administer fluids during the week prior to assessment, which affected serum creatinine. No other significant changes in BUN, phosphorus, potassium, PCV, urine specific gravity or UPC were noted.

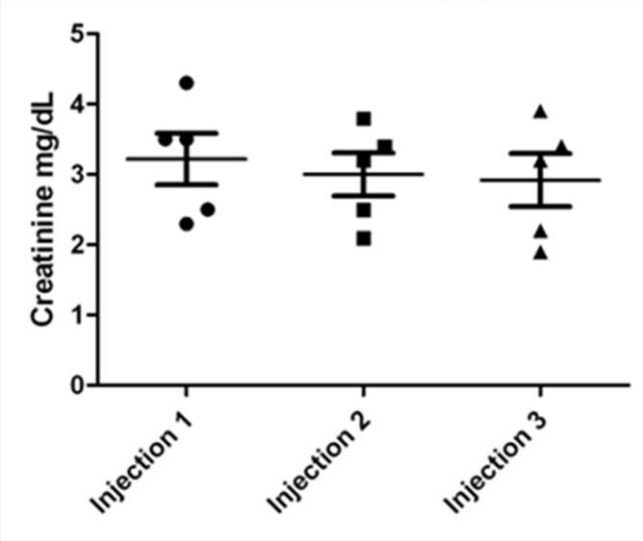



**Figure 5.7:** Serum creatinine for cats in Group A, who received three doses of 2 x $10^6$ MSC IV. A statistically significant decrease in creatinine is seen (p = 0.01).

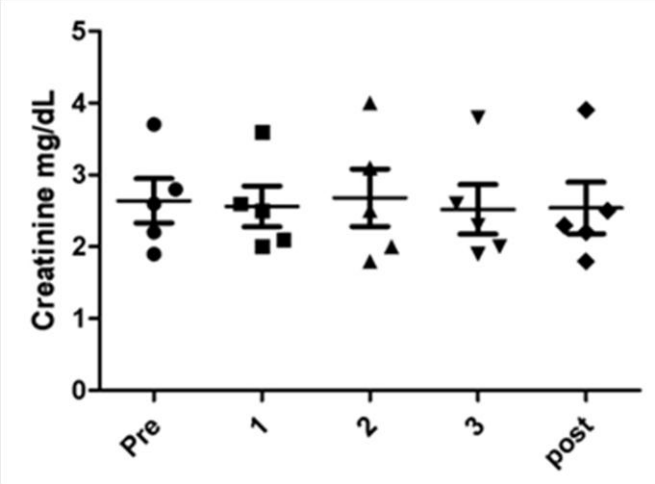



**Figure 5.8.** Serum creatinine for cats in Group B that received three doses of 4 x $10^6$ MSC IV. No significant difference in creatinine was detected.

Group B cats had an overall trend towards improvement in GFR (p = 0.056), with individual increases of (75%, 10%, 61%, 7%, 6%) compared to untreated CKD control cats (7%, -18%, -22%). (**Figure 5.9**)

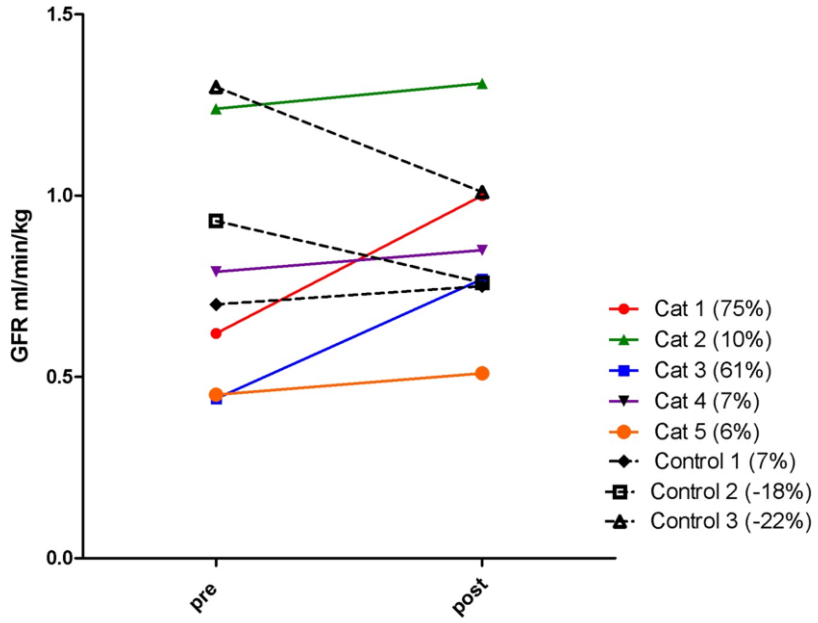



**Figure 5.9:** Iohexol GFR results at 0 and 8 weeks for 5 cats that received 4 x 106 MSC IV every 2 weeks for three treatments. Control cats had GFR performed at 0 and 8 weeks only.

### *5.4.2e Effects of MSC Administration on Urinary Cytokine Levels*

Cats in Group A had IL-6, IL-8, IL-10, TGF-β1, MCP-1 performed on urine samples. However IL-6, IL-10 and TGF-β1 were non-detectable in the urine samples. Results for MCP-1 and IL-8 are depicted in **Figures 5.10 and 5.11**. Using a repeated measures ANOVA test, a statistically significant decrease in MCP-1 (p = 0.0001) as well as in IL-8 (p = 0.01) was detected. Subsequently, for Group B cats, a multispecies TGF-β1 ELISA test was used (multispecies TGF-β1: Invitrogen, Camarillo, CA) which was more successful. Cats in Group B had IL-8, MCP-1, TGF-β1 and VEGF ELISAs performed on urine samples. No statistically significant differences in any of the cytokine levels measured for cats in Group B were detected (**Figures 5.12-5.15**).

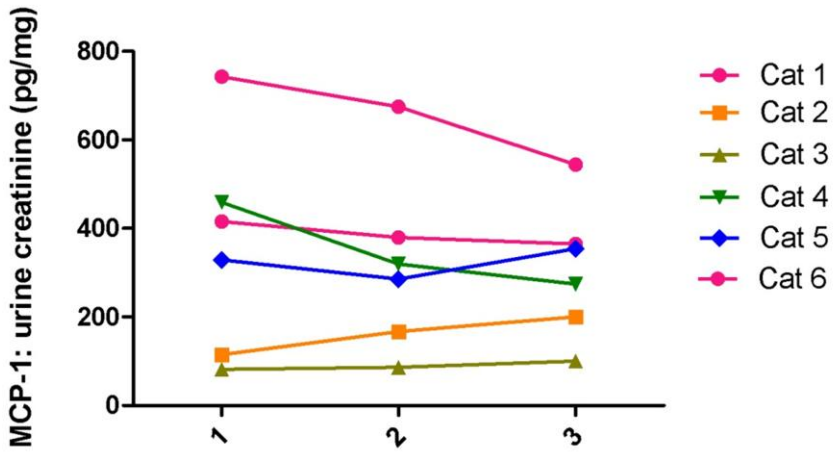



**Figure 5.10:** MCP-1:urine creatinine ratio in 6 cats from Group A that received 2 x $10^6$ MSC IV every other week for three injections. There was a statistically significant decrease in MCP-1 (p = 0.0001).

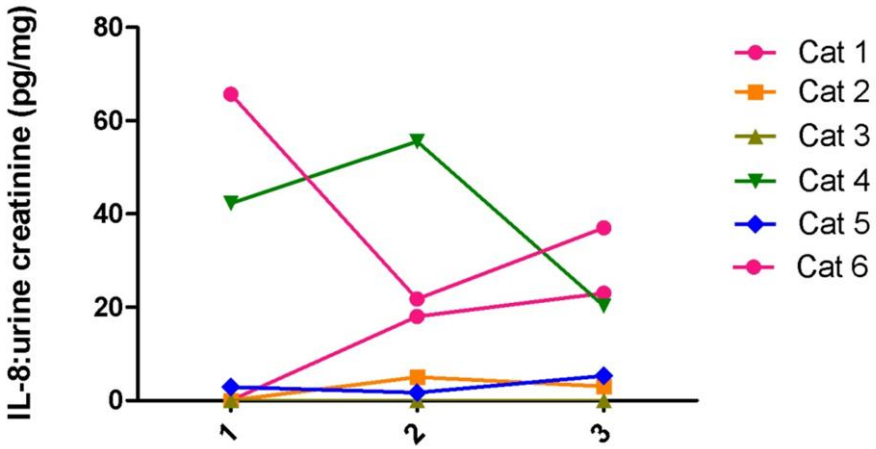



**Figure 5.11:** IL-8:urine creatinine ratio in 6 cats from Group A that received 2 x $10^6$ MSC IV every other week for three injections. There was a statistically significant decrease in IL-8 (p = 0.01).

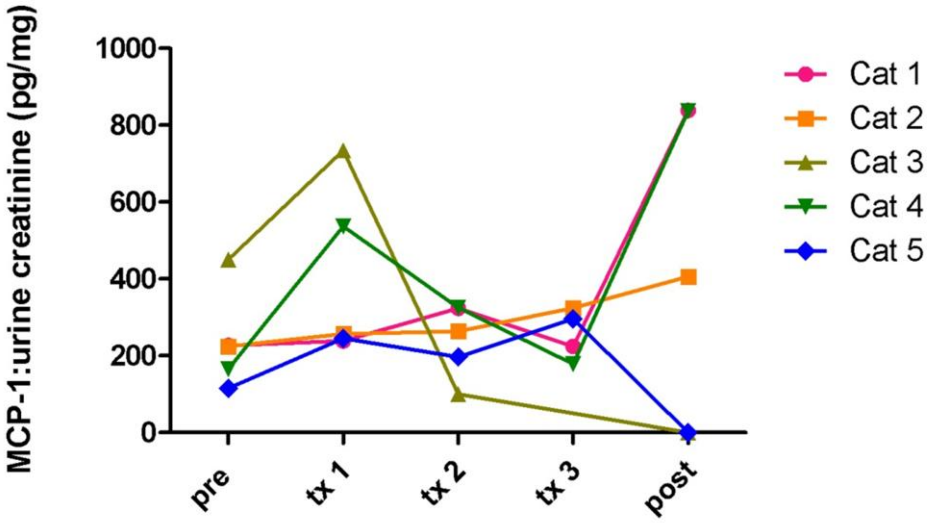



**Figure 5.12:** MCP-1:urine creatinine ratio in 5 cats from Group B that received 4 x $10^6$ MSC IV every other week for three injections. There was no statistically significant change in MCP-1.

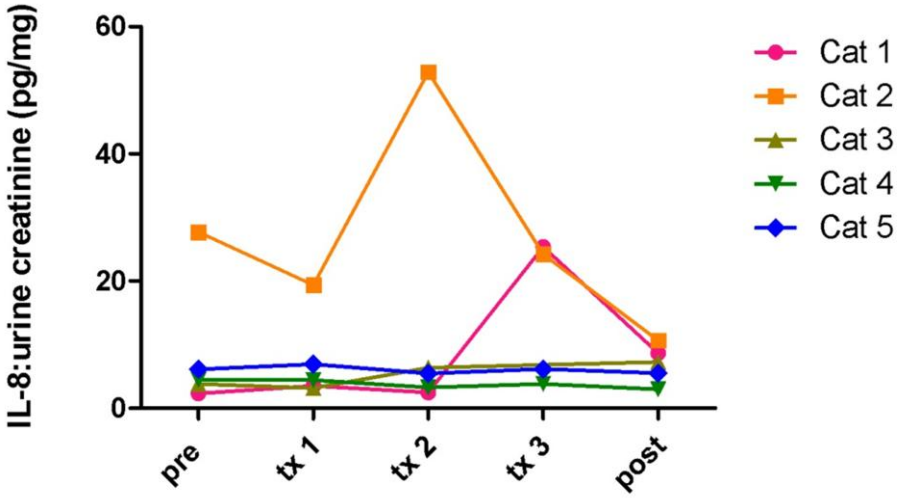



**Figure 5.13:** IL-8:urine creatinine ratio in 5 cats from Group B that received 4 x $10^6$ MSC IV every other week for three injections. There was no statistically significant change in IL-8.

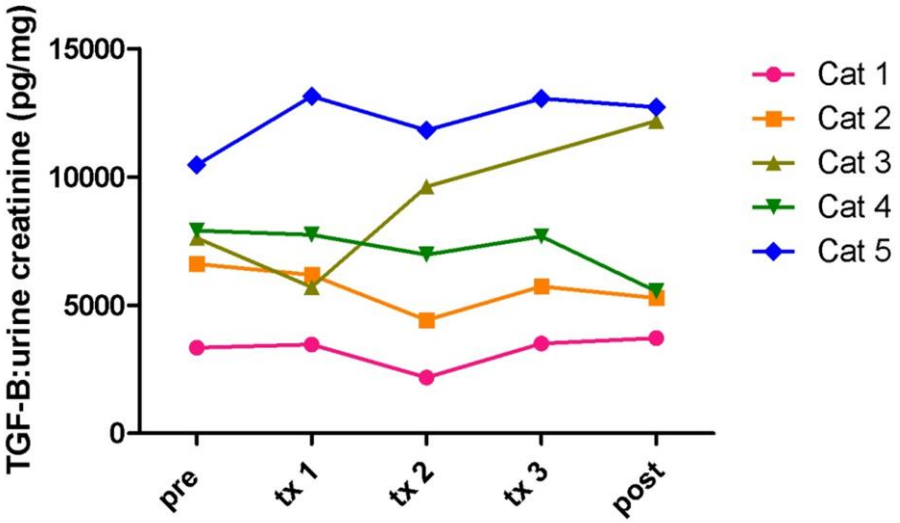



**Figure 5.14:** TGF-β1:urine creatinine ratio in 5 cats from Group B that received 4 x $10^6$ MSC IV every other week for three injections. There was no statistically significant change in TGF-β1.

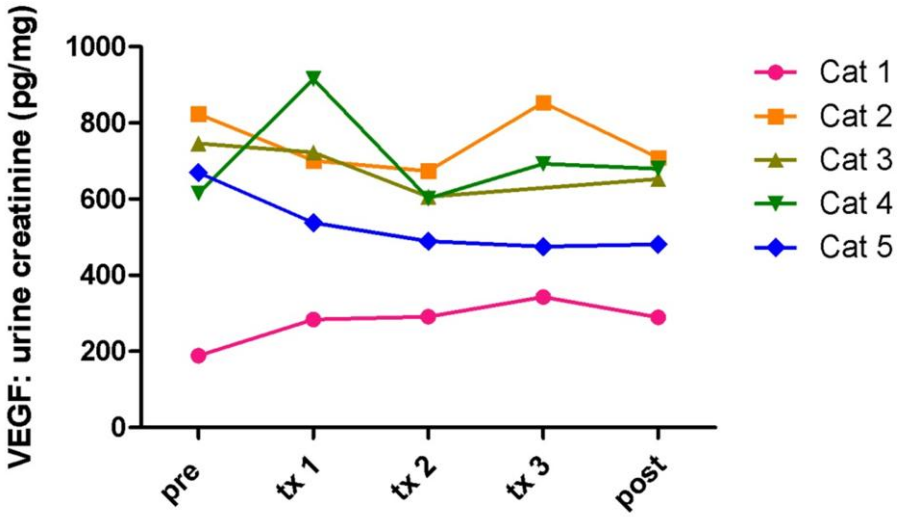



**Figure 5.15:** VEGF:urine creatinine ratio in 5 cats from Group B that received 4 x $10^6$ MSC IV every other week for three injections. There was no statistically significant change in VEGF.

## 5.5 Discussion

In this body of work, the feasibility of harvesting feline stem cells and expanding them for therapeutic use in CKD was assessed. MSC have previously been shown to be effective in ameliorating disease in rodent models of induced CKD. However MSC therapy has not been evaluated in a naturally-occurring model of CKD, such as the cat. Feline MSC were successfully harvested and expanded from bone marrow and adipose sources; the latter was preferred for ease of collection, expansion and superior yield. These studies further assessed the safety and outcome of intra-renal administration of autologous MSC in CKD cats and the outcome of intravenous administration of allogeneic cryopreserved aMSC in CKD cats. Intrarenal injection did not induce immediate or delayed adverse effects. Some cats that received aMSC experienced modest improvement in GFR and a mild decrease in serum creatinine concentration, but the number of sedations and interventions required for this method would likely preclude widespread clinical application. In the intravenous study, six cats received $2 \times 10^6$ aMSC per injection and experienced a statistically significant decrease in serum creatinine with negligible side effects. Five cats received $4 \times 10^6$ aMSC per injection and side effects included vomiting during infusion and increased respiratory rate. Variable decreases in serum creatinine, increases in GFR by iohexol clearance and changes in urinary cytokines were seen in the latter group. Efficacy of repeated intravenous injection of allogeneic cryopreserved aMSC appears inconsistent and may result in an increased incidence of side effects at higher doses.

The difficulty experienced in expanding autologous bmMSC during the intrarenal study caused us to abandon these cells in favor of aMSC for the remainder of the work described here. The use of aMSC in lieu of bmMSC is advantagous for several reasons. Collection of aMSC was relatively simple and straightforward and large numbers of MSC could be obtained from negligible amounts of adipose tissue. Age and disease status may have also reduced the ability to establish and expand bmMSC cultures to a greater degree than with aMSC cultures, a phenomenon that has been observed in other species.[28]

Though the intra-renal injection method for MSC treatment of CKD in cats was feasible and appeared safe, this approach is unlikely to be readily applicable clinically. The multiple sedations

required for obtaining bone marrow or adipose tissue samples, MSC injections, and monitoring of renal function all contributed to substantial stress for the animals that could adversely affected renal function. The owners of all three cats with CKD noted that the hospital visits and sedation events affected the normal behavior of the cats and that it was generally 1 to 2 days before the cats resumed their normal behavior. In contrast, owners of cats enrolled in the IV injection study reported little effect from the hospital visit. Therefore, despite the potential benefits of intrarenal MSC injections for CKD in cats, we believe the large number of sedations and interventions required to implement this approach would preclude widespread clinical application.

Additionally, recent literature suggests that the intrarenal injection model utilized in the first pilot study may not have been necessary, and this is a potential limitation of this study design. Current thought supports paracrine effects as the mechanism responsible for the therapeutic benefit seen in renal disease models, and that MSC do not need to be injected into the site of interest due to their migratory capabilities.[12,19,29] These theories provide a potential explanation for the mild bilateral increase in GFR seen in Cat 7; MSC could have also affected the uninjected kidney through systemic effects of secreted cytokines.

As a result of our experience with the intrarenal pilot study and the availability of compelling new literature, the second pilot study incorporated alternative MSC sources and routes of administration for treatment of cats with CKD. Compelling support for IV administration of MSC came from a recent study by Semedo et al.[12] In this study, which used a rodent model of induced CKD, it was reported that repeated IV delivery of relatively small numbers of MSC could elicit a significant positive impact on renal function and renal inflammation. The intravenous pilot study was developed based on this previously successful study in rats. Allogeneic MSC were used to eliminate the need to sedate elderly ill animals for fat collection and to facilitate treatment schedules. Allogeneic MSC have been widely used in experimental stem cell transfer investigations, including clinical trials in humans, and thus are not expected to elicit unexpected adverse effects compared to autologous MSC therapy.[2,30] Successful cryopreservation of cells has also been described.[31] Accordingly, the second pilot study was designed to

assess the most logistically-simple and patient-friendly MSC therapy for cats with CKD, i.e. administration of allogeneic cryopreserved aMSC.

In the IV allogeneic pilot study, Group A cats were monitored for improvement in kidney function by assessment of changes in serum creatinine. Based on results in rodent models of induced CKD, it was expected that some improvement in these values would be seen. Although several cats did experience an improvement in serum creatinine during the study, it is not the most sensitive measurement of renal function and several factors can influence it that are not directly related to renal function.[25] These include muscle mass and hydration status.[25,32] Decreases in weight and/or muscle mass can potentially decrease in serum creatinine, while a worsening in hydration status can increase in serum creatinine. In addition, changes to renal function may occur without any concomitant alteration in serum creatinine, although this is thought to be less true once an animal reaches moderate renal dysfunction.[25] Changes in real body weight in the study cats potentially confounded serum creatinine measurements, while changes in bladder fullness at the time of weighing potentially compromised weight values. Therefore for cats in Group B, we elected to use a more objective measure of renal function, GFR as determined by iohexol clearance, which does not require patient sedation. Although this type of GFR does not allow determination individual kidney function, measurement of individual kidney function is less important assuming MSC effects are paracrine and a systemic effect is elicited.

The response seen in the CKD cats treated IV with aMSC was not as impressive as that seen in rodent models and side effects were more evident in the high dose group. Although the specific reasons for the increased incidence of side effects is not known, it is likely related to differences between the low and high dose group. The type of infusion was changed from a relatively short manual push to administration by syringe pump which involved the addition of a long plastic infusion line. At the time of administration of cells to the last enrolled cat, it was noted that stem cells adhered in the infusion line. Therefore it is possible that cats did not receive the appropriate cell number and/or cells adhered, and then detached in clumps causing respiratory signs secondary to pulmonary cell thrombosis. Secondly, it is also possible that despite extensive cell washing some aspect of the cryopreserved cells caused a reaction in

the cats, particularly in the cats receiving the higher dose. We find it unlikely that it was the larger cell numbers alone that caused the increased side effects, as large quantities of fresh MSC have been infused in cats with respiratory disease without incident (C. Reinero, personal communication). Regardless of the cause for the increased incidence of side effects and despite the logistic ease of allogeneic cryopreserved MSC, both efficacy and side effects of the pilot study were not acceptable for clinical application in feline CKD.

There may be several reasons why our assessment of the efficacy of intravenous administration of adipose-derived allogeneic cryopreserved MSC in improving renal function in CKD cats has not been as promising as that described in rodent models of induced CKD.[11-13] There is potentially a great deal of difference between rodent models involving induced CKD and the naturally-occurring disease present in the cats used in this study. Many rodent models involve acute insult to the kidney with administration of MSC within a few weeks, a relatively short time after renal insult.[12,33] MSC therapy has been shown to be very beneficial in models of acute renal failure, and therefore the time frame of MSC administration may represent a more acute rather than chronic disease process. Additionally, the induced disease models may not represent the changes occurring in a truly chronic, naturally-occurring disease process.[4] Cats have an extended lifespan and can have CKD for months to years prior to enrollment in a clinical trial. The inherent differences between the induced rodent models and naturally-occurring feline CKD highlight reasons why cats are potentially an excellent translational model for the study of MSC therapy in CKD. A second potential reason for variable efficacy in the IV pilot studies is the use of allogeneic cells. Although allogeneic MSC are immune privileged and are not expected to incite an immune response, they may not be as effective as autologous cells.[30] Allogeneic MSC may be cleared faster from the body in comparison to autologous cells, which could influence efficacy. However it has not been determined how long the cells would need to be present to effectively induce immunomodulatory paracrine effects. Decreased efficacy of allogeneic in comparison to autologous MSC has been shown in one acute renal failure rodent study.[30] Lastly, cryopreservation may decrease the immunosuppressive potential of MSC. Previous studies assessing the effects of cryopreservation on MSC have demonstrated no change in

characterization, but have not assessed immune function.[31,34] Preliminary data from our laboratory implies that immunomodulatory capabilities may be altered in cryopreserved feline aMSC. This topic merits further investigation and future studies should likely focus on the use of fresh non-cryopreserved MSC.

In conclusion, the pilot studies described above demonstrate the feasibility of obtaining feline MSC from adipose tissue for use in clinical trials. Specifically the use of MSC for the amelioration of feline CKD was assessed with both an intra-renal injection model and an IV injection model. Intrarenal injection of autologous MSC resulted in mild improvement of GFR and creatinine in a small number of cats but was deemed logistically challenging for the clinical setting. A more clinically feasible model, IV injection of allogeneic cryopreserved cells, showed variable effects on renal function and adverse effects were noted at higher doses of MSC. The use of MSC for treatment of feline CKD shows potential, although additional studies are needed to explore how efficacy can be improved.

## 5.7 References


**1.** Martin DR, Cox NR, Hathcock TL, Niemeyer GP, Baker HJ. Isolation and characterization of multipotential mesenchymal stem cells from feline bone marrow. *Exp Hematol.* Aug 2002;30(8):879-886.

**2.** McTaggart SJ, Atkinson K. Mesenchymal stem cells: immunobiology and therapeutic potential in kidney disease. *Nephrology (Carlton).* Feb 2007;12(1):44-52.

**3.** Reinders ME, Fibbe WE, Rabelink TJ. Multipotent mesenchymal stromal cell therapy in renal disease and kidney transplantation. *Nephrol Dial Transplant.* Jan 2010;25(1):17-24.

**4.** Little MH, Rae FK. Review article: Potential cellular therapies for renal disease: can we translate results from animal studies to the human condition? *Nephrology (Carlton).* Sep 2009;14(6):544-553.

**5.** Chhabra P, Brayman KL. The use of stem cells in kidney disease. *Curr Opin Organ Transplant.* Feb 2009;14(1):72-78.

**6.** Choi SJ, Kim JK, Hwang SD. Mesenchymal stem cell therapy for chronic renal failure. *Expert Opin Biol Ther.* Aug 2010;10(8):1217-1226.

**7.** Asanuma H, Meldrum DR, Meldrum KK. Therapeutic applications of mesenchymal stem cells to repair kidney injury. *J Urol.* Jul 2010;184(1):26-33.

**8.** O'Brien T, Barry FP. Stem cell therapy and regenerative medicine. *Mayo Clin Proc.* Oct 2009;84(10):859-861.

**9.** Zerbini G, Piemonti L, Maestroni A, Dell'Antonio G, Bianchi G. Stem cells and the kidney: a new therapeutic tool? *J Am Soc Nephrol.* Apr 2006;17(4 Suppl 2):S123-126.

**10.** Zubko R, Frishman W. Stem cell therapy for the kidney? *Am J Ther.* May-Jun 2009;16(3):247-256.

**11.** Lee SR, Lee SH, Moon JY, et al. Repeated administration of bone marrow-derived mesenchymal stem cells improved the protective effects on a remnant kidney model. *Ren Fail.* 2010;32(7):840-848.

**12.** Semedo P, Correa-Costa M, Antonio Cenedeze M, et al. Mesenchymal stem cells attenuate renal fibrosis through immune modulation and remodeling properties in a rat remnant kidney model. *Stem Cells.* Dec 2009;27(12):3063-3073.

**13.** Villanueva S, Ewertz E, Carrion F, et al. Mesenchymal stem cell injection ameliorates chronic renal failure in a rat model. *Clin Sci (Lond).* Dec 2011;121(11):489-499.

**14.** Cavaglieri RC, Martini D, Sogayar MC, Noronha IL. Mesenchymal stem cells delivered at the subcapsule of the kidney ameliorate renal disease in the rat remnant kidney model. *Transplant Proc.* Apr 2009;41(3):947-951.

**15.** Ninichuk V, Gross O, Segerer S, et al. Multipotent mesenchymal stem cells reduce interstitial fibrosis but do not delay progression of chronic kidney disease in collagen4A3-deficient mice. *Kidney Int.* Jul 2006;70(1):121-129.

**16.** Morigi M, Imberti B, Zoja C, et al. Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure. *J Am Soc Nephrol.* Jul 2004;15(7):1794-1804.

**17.** Kim SS, Park HJ, Han J, et al. Improvement of kidney failure with fetal kidney precursor cell transplantation. *Transplantation.* May 15 2007;83(9):1249-1258.

**18.** Kitamura S, Yamasaki Y, Kinomura M, et al. Establishment and characterization of renal progenitor like cells from S3 segment of nephron in rat adult kidney. *Faseb J.* Nov 2005;19(13):1789-1797.

**19.** Togel F, Weiss K, Yang Y, Hu Z, Zhang P, Westenfelder C. Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. *Am J Physiol Renal Physiol.* May 2007;292(5):F1626-1635.

**20.** Togel F, Zhang P, Hu Z, Westenfelder C. VEGF is a mediator of the renoprotective effects of multipotent marrow stromal cells in acute kidney injury. *J Cell Mol Med.* Aug 2009;13(8B):2109-2114.
**21.** Avery PR, Lehman TL, Hoover EA, Dow SW. Sustained generation of tissue dendritic cells from cats using organ stromal cell cultures. *Vet Immunol Immunopathol.* Jun 15 2007;117(3-4):222-235.
**22.** Kolf CM, Cho E, Tuan RS. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. *Arthritis Res Ther.* 2007;9(1):204.
**23.** Locke M, Windsor J, Dunbar PR. Human adipose-derived stem cells: isolation, characterization and applications in surgery. *ANZ J Surg.* Apr 2009;79(4):235-244.
**24.** Reger RL, Tucker AH, Wolfe MR. DIfferentiation and characterization of human MSCs. In: Prockop DJ, Phinney, D.J., Bunnell, B.A., ed. *Methods in molecular biology; mesenchymal stem cells: methods and protocols*. Totowa, NJ: Humana Press; 2008.
**25.** Kerl ME, Cook CR. Glomerular filtration rate and renal scintigraphy. *Clin Tech Small an P.* Feb 2005;20(1):31-38.
**26.** Arata S, Ohmi A, Mizukoshi F, et al. Urinary transforming growth factor-beta1 in feline chronic renal failure. *J Vet Med Sci.* Dec 2005;67(12):1253-1255.
**27.** Chakrabarti S, Syme HM, Elliott J. Urinary vascular endothelial growth factor as a marker of renal hypoxia in cats. *J Vet Int Med.* 2011;25:720.
**28.** Stenderup K, Justesen J, Clausen C, Kassem M. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone.* Dec 2003;33(6):919-926.
**29.** Togel F, Yang Y, Zhang P, Hu Z, Westenfelder C. Bioluminescence imaging to monitor the in vivo distribution of administered mesenchymal stem cells in acute kidney injury. *Am J Physiol Renal Physiol.* Jul 2008;295(1):F315-321.
**30.** Togel F, Cohen A, Zhang P, Yang Y, Hu Z, Westenfelder C. Autologous and allogeneic marrow stromal cells are safe and effective for the treatment of acute kidney injury. *Stem Cells Dev.* Apr 2009;18(3):475-485.
**31.** Martinello T, Bronzini I, Maccatrozzo L, et al. Canine adipose-derived-mesenchymal stem cells do not lose stem features after a long-term cryopreservation. *Res Vet Sci.* Aug 2011;91(1):18-24.
**32.** Baxmann AC, Ahmed MS, Marques NC, et al. Influence of muscle mass and physical activity on serum and urinary creatinine and serum cystatin C. *Clin J Am Soc Nephrol.* Mar 2008;3(2):348-354.
**33.** Choi S, Park M, Kim J, Hwang S, Park S, Lee Y. The role of mesenchymal stem cells in the functional improvement of chronic renal failure. *Stem Cells Dev.* Apr 2009;18(3):521-529.
**34.** Ginis I, Grinblat B, Shirvan MH. Evaluation of Bone Marrow-Derived Mesenchymal Stem Cells After Cryopreservation and Hypothermic Storage in Clinically Safe Medium. *Tissue Eng Part C Methods.* Feb 2 2012.

# CHAPTER 6: CONCLUDING REMARKS

## 6.1 Significance of Work

With better care, the average lifespan of our feline companions has increased, and geriatric medicine becomes more and more a focus of veterinary care. As CKD is so common in the geriatric cat population, advancements in therapeutic options have the potential to benefit a great number of cats. The work presented in this dissertation is intended to further our knowledge of possible etiologies of CKD and to develop novel treatment strategies to help cats afflicted with this disease. In Chapter 3 we demonstrated an association between telomere length, cellular senescence and CKD, which provides valuable insight into pathogenesis of aging in the kidney and its propensity for disease and may provide a foundation for novel treatment strategies. In Chapter 4 we determined the feline pharmacokinetics and pharmacodynamics of mirtazapine, a novel appetite stimulant, and demonstrated its efficacy in increasing appetite in normal cats and cats with CKD. This will potentially improve the nutrition and quality of life of cats with CKD. In Chapter 5 we investigated the use of mesenchymal stem cell therapy as a novel treatment for feline CKD. Although results were variable, this particular therapeutic strategy has great potential and should be explored further.

End stage renal disease is also an increasingly common diagnosis in humans. Feline CKD is one of very few naturally occurring models of kidney disease, and the pathology is very similar in many respects to the human disease process. Therefore information gathered in the course of studying this disease in cats has tremendous translational research potential that could be of benefit to humans.

## 6.2 Specific Aim 1 (Chapter 3: Telomeres)

A leading hypothesis of aging suggests that as cellular senescence increases, organ function declines. There is preliminary information from other investigators that telomeres in cats do indeed shorten with increasing age,[1] thus implicating telomere shortening in the feline aging process. However, no attempt has been made to correlate telomere length or other markers of cellular senescence with any disease process in cats. The specific aim for this chapter was to investigate the role of telomere length and cellular senescence in feline CKD. We demonstrated that significantly shorter telomeres are found in the renal proximal and distal tubular cell population of CKD cats compared to young normal or geriatric normal cats. There was no difference between CKD cats and normal cats when liver or skin telomere length was measured. Additionally increased cell senescence was observed in the kidney of CKD cats in comparison to young normal cats. CKD cats tended to increased senescence in comparison to normal geriatric cats, but this finding did not reach statistical significance. Curiously, neither telomere length nor cellular senescence were correlated with age, but the normal geriatric population available for assessment was small. Additional studies with larger numbers of cats will be necessary to confirm if there is an effect on telomere length and cellular senescence from age alone. Unfortunately this is hampered by the difficulty of finding geriatric cats who are not affected by CKD.

Although these associations do not necessarily demonstrate a cause and effect relationship, they do reveal that telomere dysfunction may indeed contribute to predisposition for development of CKD. It is also possible that additional unknown factors predispose cats to renal insult and the resulting continued need for repair and replication in this organ leads to telomere shortening. Further studies will be necessary to explore these interrelated factors. Regardless, the association between telomere length, cellular senescence and CKD provides a foundation of knowledge for development of additional treatment strategies.

Another potentially exciting area of future telomere research that stems from the current work is exploration of the possibility that normal feline kidneys have telomeric aggregates in the renal proximal

tubular cell population. We demonstrated the presence of unusually large telomeric signals in this cell population. If these are indeed shown to be telomeric aggregates, then the possibility exists that telomeric dysfunction predisposes cats to CKD, which would be a pivotal finding.

The technique adapted for use in assessing telomeres in the feline kidney, TELI-FISH, also has the potential to be useful for additional research studies. Telomere shortening may occur as a result of varied insults upon the kidney, such as hypoxia, oxidative stress, etc. Telomere length could be used as a tool to monitor the effectiveness of therapeutic interventions to ameliorate this damage.[2] It could also be used to monitor renal health in other ways, such as the effect of renal transplantation methods on the donor kidney.[3] In conclusion, the demonstration of an association between telomere length, cellular senescence and feline CKD has opened up exciting new avenues of research and diagnostic capabilities for studying CKD in cats and humans.

## 6.3 Specific Aim 2 (Chapter 4: Mirtazapine)

Poor body condition is associated with a poorer prognosis in CKD.[4] In additional, poor appetite has a negative effect on owner perception of quality of life and can result in a great deal of emotional stress, as well as ultimately leading to euthanasia. Nutrition is therefore important for long term prognosis and efforts to directly target nausea and appetite, in addition to other metabolic medical therapies will undoubtedly benefit patients. New pharmacological options for the management of inappetence in CKD cats would be of great interest to the veterinary community. Mirtazapine is an appetite stimulant and anti-nausea medication that has recently gained popularity in veterinary medicine and anecdotally appears to be helpful for the management of appetite. However, previously no pharmacokinetic information existed on the drug in cats.

The focus of this chapter was the determination of the pharmacokinetics and pharmacodynamics of commonly prescribed doses of mirtazapine in normal cats, elderly cats and cats with CKD, and then ultimately a placebo-controlled blinded crossover clinical trial to assess the efficacy of mirtazapine in

CKD cats. The overall aim was to provide information to further guide the use of mirtazapine as an appetite stimulant in cats, specifically for those suffering from CKD. We assessed the hypothesis that mirtazapine is clinically useful for the management of feline CKD, and results in increased appetite and weight gain. This body of work demonstrated that there are differences in the metabolism of mirtazapine between young normal cats and CKD cats. Young cats could receive daily mirtazapine at a low dose without significant likelihood of drug accumulation whereas CKD cats should receive the drug every other day. In a subsequent clinical trial, mirtazapine significantly increased appetite, activity and weight in CKD cats when administered every other day for three weeks. Additionally, a significant decrease in vomiting was noted. This demonstrated that mirtazapine does have significant appetite stimulating and anti-nausea effects in CKD cats. In conclusion the information gathered in this body of work will help clinicians prescribe mirtazapine more effectively with a decreased incidence of unwanted drug side effects. Most importantly, it will help improve the quality of life and potentially prognosis of cats suffering from CKD.

**6.4 Specific Aim 3 (Chapter 5: Stem Cells)**

In both cats and humans, CKD is characterized by tubulointerstitial damage, fibrosis and progressive loss of renal function, and is commonly described as the final common pathway after any one of multiple types of renal insults. In several rodent CKD models, administration of MSC has been beneficial, especially with respect to reducing intra-renal inflammation and suppressing fibrosis.[5-8] In vitro studies have demonstrated that MSC can produce growth factors, cytokines, and anti-inflammatory mediators, all of which could help maintain or improve renal function and suppress intra-renal inflammation.[9-11] At present, however, there is little published work regarding the use of MSC for treatment of naturally occurring CKD, such as feline CKD.

The overall aim of this chapter was the evaluation of MSC therapy as a novel treatment strategy for cats with CKD. The hypothesis for specific aim 3 was that injection of MSC would not be harmful

and would improve renal function in CKD cats due to anti-inflammatory effects. A series of pilot studies was used to test this hypothesis; a pilot study of intrarenal injection of autologous stem cells and two pilot studies of intravenously injected allogeneic cryopreserved MSC. Minimum database, GFR and urinary cytokines were measured to assess intra-renal inflammation, fibrosis and vascular health and the possible effects of MSC injection on these factors. We determined that MSC could be successfully harvested and cultured from bone marrow and adipose sources, but the latter was preferred for ease of collection, expansion and superior yield. Intrarenal injection did not induce immediate or longer-term adverse effects. Two CKD cats that received intrarenal adipose-derived MSC experienced modest improvement in GFR and a mild decrease in serum creatinine concentration. In the allogeneic cryopreserved intravenous study, six cats received $2 \times 10^6$ MSC per injection and experienced a significant decrease in serum creatinine with negligible side effects. Five cats received $4 \times 10^6$ MSC per injection and side effects included vomiting during infusion and increased respiratory rate. Variable decreases in serum creatinine, increases in GFR by iohexol clearance and changes in urinary cytokines were seen. Despite the mild improvement in creatinine seen in some of the cats, none had improvement to the extent described in rodent models. There are multiple variables that need to be explored in future studies to determine why this would be. Factors affecting efficacy could include cryopreservation, dose, administration method or ultimately the difference between an induced model of CKD vs. a naturally occurring one. In conclusion, while MSC therapy potentially holds promise for palliation of CKD, a additional work is necessary to determine if this therapy can be manipulated to increase efficacy. The work described in this dissertation has provided a strong foundation for further studies.

## 6.5 References


**1.** McKevitt TP, Nasir L, Wallis CV, Argyle DJ. A cohort study of telomere and telomerase biology in cats. *Am J Vet Res.* Dec 2003;64(12):1496-1499.

**2.** Houben JM, Moonen HJ, van Schooten FJ, Hageman GJ. Telomere length assessment: biomarker of chronic oxidative stress? *Free Radic Biol Med.* Feb 1 2008;44(3):235-246.

**3.** Joosten SA, van Ham V, Nolan CE, et al. Telomere shortening and cellular senescence in a model of chronic renal allograft rejection. *Am J Pathol.* Apr 2003;162(4):1305-1312.

**4.** Parker VJ, Freeman LM. Association between body condition and survival in dogs with acquired chronic kidney disease. *J Vet Intern Med.* Nov-Dec 2011;25(6):1306-1311.

**5.** Semedo P, Correa-Costa M, Antonio Cenedeze M, et al. Mesenchymal stem cells attenuate renal fibrosis through immune modulation and remodeling properties in a rat remnant kidney model. *Stem Cells.* Dec 2009;27(12):3063-3073.

**6.** Ninichuk V, Gross O, Segerer S, et al. Multipotent mesenchymal stem cells reduce interstitial fibrosis but do not delay progression of chronic kidney disease in collagen4A3-deficient mice. *Kidney Int.* Jul 2006;70(1):121-129.

**7.** Lee SR, Lee SH, Moon JY, et al. Repeated administration of bone marrow-derived mesenchymal stem cells improved the protective effects on a remnant kidney model. *Ren Fail.* 2010;32(7):840-848.

**8.** Villanueva S, Ewertz E, Carrion F, et al. Mesenchymal stem cell injection ameliorates chronic renal failure in a rat model. *Clin Sci (Lond).* Dec 2011;121(11):489-499.

**9.** Togel F, Weiss K, Yang Y, Hu Z, Zhang P, Westenfelder C. Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. *Am J Physiol Renal Physiol.* May 2007;292(5):F1626-1635.

**10.** McTaggart SJ, Atkinson K. Mesenchymal stem cells: immunobiology and therapeutic potential in kidney disease. *Nephrology.* Feb 2007;12(1):44-52.

**11.** Barry FP, Murphy JM, English K, Mahon BP. Immunogenicity of adult mesenchymal stem cells: lessons from the fetal allograft. *Stem cells and development.* Jun 2005;14(3):252-265.