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AUTHORS: R.E. Jepson

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Title: Current understanding of the pathogenesis of progressive chronic kidney disease in cats

Author: Rosanne E. Jepson

BVSc MVetMed PhD DipACVIM DipECVIM-CA FHEA MRCVS

rjepson@rvc.ac.uk

Corresponding address:

Royal Veterinary College

Hawkshead Lane

North Mymms

Herts

AL9 7TA

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Abstract:

In cats with chronic kidney disease (CKD), the most common histopathological finding is tubulointerstitial inflammation and fibrosis. However, these changes reflect a non-specific response of the kidney to any inciting injury. The risk of development of CKD in a patient is likely to reflect genetic predispositions, ageing, environmental and individual factors that influence decline in renal function over the course of a cat's life. However, there is still relatively little information about exactly which risk factors predispose a cat to develop CKD and these will be explored. Whilst many cats diagnosed with CKD have stable disease for many years, some cats show overtly progressive disease. From the human and experimental literature the pathophysiological processes underpinning this progression have been elucidated and include haemodynamic alterations after loss of functional renal mass, activation of the renin-angiotensin aldosterone system, proteinuria, alteration in phosphorus homeostasis, hypoxia and oxidative stress. Many of these pathophysiological mechanisms are likely to translate to feline CKD and an understanding of their role in the progression of disease gives the opportunity for therapeutic intervention.

Key points:

- **Chronic kidney disease (CKD) is a common condition in mature, senior and geriatric cats, which is characterized by tubulointerstitial inflammation and fibrosis.**
- **CKD is a complex disease condition, the development of which is likely to be influenced by genetic, environmental and individual patient factors.**

- **Factors that have been associated with progressive CKD from experimental and human medicine include haemodynamic adaptations to renal injury, systemic hypertension, activation of the renin-angiotensin aldosterone system, proteinuria, hyperphosphatemia hypoxia and oxidative stress.**
- **Irrespective of the inciting injury, the common pathway for the progression of renal injury is via inflammation and fibrosis.**
- **Understanding factors associated with progression of disease gives potential for therapeutic intervention, which may slow advancing disease.**

Introduction: Chronic kidney disease (CKD) is a common condition identified in cats at both general practice and referral level. The term CKD is used to imply alteration in structure or function of the kidney that has occurred over a period of time, typically 3 months. A number of different underlying renal diseases can affect the feline kidney, some localizing to a particular region of the kidney, some being congenital and others acquired in origin. At least initially, not all of these conditions will result in azotemia and yet they may still fulfill the criteria of CKD e.g. altered tubular function or primary glomerular disease. However, when examining post-mortem tissue from geriatric cats diagnosed with CKD in a first-opinion setting, where the predominant breed examined was either the domestic short or longhair, then specific renal diseases accounted for only ~16% of all CKD.¹ The most common histopathological finding was non-specific tubulointerstitial inflammation, fibrosis and mineralization referred to as tubulointerstitial nephritis.¹⁻⁴ The development and progression of these lesions

will be the focus of this article but given the limited response of the kidney to an inciting injury, tubulointerstitial inflammation and fibrosis is a common end pathology for many primary renal diseases.

Early studies suggested that approximately 15-30% of cats over the age of 15 years show evidence of CKD.⁵ However, markers of GFR used in clinical practice are insensitive for the detection of early decline in renal function (Chapters 1 and 2) and it is appreciated that substantial renal pathology may be present by the time a patient develops azotemic CKD.² Such patients may have other evidence that points to the clinical diagnosis of CKD without the requirement for renal biopsy e.g. persistently inadequately concentrated urine or abnormalities identified with diagnostic imaging of the kidney. These patients are recognized within the International Renal Interest Society (IRIS) staging system as having non-azotemic stage 1 or 2 CKD. Recent studies that include these early diagnosed patients suggest that the prevalence of CKD is much higher, and in the study by Marino and colleagues 80% of cats > 15 years were defined as having CKD.⁶

Aetiology of feline chronic kidney disease:

The underlying aetiology of feline CKD, where the primary histopathological finding is tubulointerstitial nephritis, is poorly understood. CKD should be considered as a complex disease that is likely to be influenced by genetic, individual, and environmental factors.

Ageing and the kidney

In human medicine it is known that GFR declines with age as a consequence of renal structural change, tubular dysfunction and a decrease in the number of functioning nephrons. These changes begin around the age of 30-40 years but accelerate after the age of 50-60 years.⁷⁻⁹ Decline in GFR in humans has been reported to be between 0.4 and 1.02 ml/min/year and CKD to be present in approximately 35% of the general population over the age of 70 years using current criteria.^{7,10} This has led studies to question whether the criteria that are used for the diagnosis and staging of CKD (Kidney Disease Outcomes Quality Initiative; KDOQI guidelines) should be applied equally to young and old alike given that decrease in GFR may be 'normal' in old age and the mortality risk associated with a given stage of CKD may be different between young and older age groups. It can therefore be debated whether a diagnosis of CKD in the elderly truly represents a disease process rather than part of normal ageing.¹⁰⁻¹³

Although CKD is most often identified in cats over the age of 12 years, there is evidence that tubulointerstitial inflammation may be identified in the renal parenchyma of young cats that have died for other reasons and as such it has been proposed that the development of CKD may also be part of a normal aging process in cats.¹⁴

Ageing is a programmed biological process, which is regulated by many genes. It results in impairment to normal adaptive responses and homeostatic mechanisms that makes organs susceptible to either internal or external stressors.¹⁰ Transcriptional differences have been identified between the young and the old affecting many genes.¹⁰ However, there are certain genes, e.g. *Klotho* (an ageing suppressor gene encoding for alpha-Klotho) and *SIRT 1* (encoding for

sirtuin-1 which is a NAD-dependent histone deacetylase involved in cellular regulation), which may be of particular interest.^{10,15} Klotho was first identified in 1997 in a mutant mouse strain that demonstrated an ageing phenotype and shortened lifespan.¹⁶ Subsequently its concurrent role in phosphorus homeostasis and kidney disease has made it a gene and molecule of particular interest with relation to CKD and declining renal function with age.^{17,18}

A number of different mechanisms can contribute to age related organ dysfunction including mitochondrial injury, telomere shortening, oxidative stress, pro-fibrogenic and pro-inflammatory mediators and an imbalance between cell repair and proliferation versus apoptosis and cell death (Figure 1). However, many of these mechanisms occur not only during ageing but also as part of an organ's response to injury and as part of a healing process. Cellular senescence occurs as part of the aging process (replicative senescence) and refers to the situation where cells enter a state of replication and growth arrest.¹¹ Such cells remain viable but show an altered morphology including increased expression of senescence associated β -galactosidase (SABG), accumulation of lipofuscin granules, lack of response to mitogenic stimuli and in some species, e.g. humans, replicative senescence is associated with telomere shortening and reduced telomerase activity.¹⁰ Cellular senescence can also be stimulated by a number of physiologic stressors, e.g. oxidative stress, mitochondrial damage, renin-angiotensin system (RAAS) activation, which is referred to as stress-induced premature senescence (SIPS). These stressors may also contribute to telomere shortening.¹¹ Cellular senescence markers e.g. telomere shortening, SABG and P16^{INK4a} (involved in the SIPS pathway), have

been shown to correlate with renal ageing in humans although there are inter-species differences.¹⁹⁻²¹ Senescent cells have altered secretion of products such as transforming growth factor- β (TGF β), epithelial growth factor, insulin like growth factor and vascular endothelial growth factor (VEGF). The net effect of these changes is reduced capacity of the kidney to respond to repair and withstand normal stressors and also reducing it's ability to recover from periods of, for example, ischemic injury and promotion of inflammation and fibrosis.¹¹

In cats, preliminary data evaluating telomere shortening supports the concept of an ageing process. Telomere length has been evaluated in renal (proximal (PTC) and distal tubule), hepatic and dermal tissue from cats with CKD, geriatric and young control cats by telomere fluorescence in-situ hybridization with immunostaining (TELI-FISH) in addition to evaluation of SABG.²² Telomere shortening was evident in PTC of geriatric cats with CKD compared to age matched and young controls despite no difference in telomere length in skin or liver from the same groups.²² Significantly increased staining for SABG was also found in renal tissue from cats with CKD compared to the young controls although the difference between geriatric control cats was not significant.²² It therefore seems feasible that aging may be a component of the decline in renal function that we see in older cats but it is also likely that other individual and environmental factors contribute to an individual's overall risk of developing CKD.

Association of chronic kidney disease with demographic, environmental and individual factors:

There are relatively few clinical studies that have evaluated phenotypic, environmental or lifestyle risk factors for the development of feline CKD. However, a recent study indicated that poor body condition, periodontal disease, cystitis, being male neutered rather than female spayed and anaesthesia or documented dehydration in the preceding year were risk factors for CKD.²³ It has been suggested that certain breeds of cats may be predisposed to CKD e.g. Persian, Abyssinian, Siamese, Ragdoll, Maine coon but the current evidence base for these breed predispositions is low.^{5,24,25} Similarly although the study above suggested a predisposition in neutered male cats, overall other studies have not supported a sex predisposition.

Concern has previously been raised regarding the role that diet may play in the development of CKD in cats. A potassium depleted high protein diet has previously been associated with the development of kidney disease whilst a 2 year study indicated no association between high salt intake and adverse effect on renal function in older cats.^{26,27} A single study has suggested that ad-lib feeding and increased ash intake were associated with CKD compared to control cats although the study was relatively small.²⁸ The study by Greene and colleagues did not support an association between diet and development of CKD such that overall evidence is controversial and further work is required.

There are many interventions that occur over a cat's lifetime that could impact on the future development of CKD; for example exposure to nephrotoxic drugs or renal toxins, periods of pre-renal azotemia and the requirement for general anaesthesia. Such episodes may reflect periods of undetected acute kidney injury (AKI), which may be a stimulus for inflammation and fibrosis (Chapter 3). It can

be hypothesised that, throughout a cat's lifetime, serial small AKI events could lead to an increased risk of developing CKD.

One further frequent intervention that has been investigated as a potential trigger for the development of CKD is vaccination. For vaccine manufacture, feline viruses (Feline Herpes virus-1, calicivirus and panleukopenia virus) are initially propagated using an immortal line of feline derived tubular epithelial cells, Crandell-Rees feline kidney cells (CRFK). It is impossible for all antigenic components of these cells to be extracted during vaccine purification and manufacture and therefore exposure to antigenic components may occur.

Administration of certain vaccines could therefore be hypothesized to stimulate antibody production, which may bind feline renal proteins and initiate an inflammatory response. This hypothesis was explored in a series of studies where cats were exposed to either CRFK cell lysate or FRCVP vaccination.²⁹

Young cats demonstrated antibody response to both parenteral administration of FRCVP vaccines and CRFK cell lysate but there was no histopathological evidence of renal disease after 56 weeks of study.²⁹ A follow up study, evaluated repeated exposure of previously sensitized cats to CRFK lysate. Although this study was small there was evidence of tubulointerstitial inflammation in 3/6 cats repeatedly inoculated with the CRFK lysate.³⁰ The antigens have been identified as α -enolase and annexin-A2.³¹ However, to date there have been no published epidemiological studies that have evaluated vaccination as a risk factor either for the development or progression of CKD and any causality between vaccination and naturally occurring CKD remains to be determined.

Association of the development of chronic kidney disease with concurrent

disease:

In human medicine there are a number of disease conditions, which are known to increase an individual's risk of developing CKD e.g. cardiovascular disease, diabetes mellitus and systemic hypertension. However, the evidence base for concurrent disease influencing the development and progression of CKD in cats is much more limited.

- **Cardiovascular and renal disorder:** The term cardiorenal syndrome has been coined in human medicine to express the relationship that exists between **cardiovascular** and renal disease and to define the situation whereby acute or chronic dysfunction in one organ may induce acute or chronic dysfunction in the other.³² Both cardiac and renal disease are common in cats and therefore these conditions may be diagnosed concurrently. However, there has been very little focus on the interplay between these two body systems, which depend on many similar homeostatic mechanisms. The role that cardiovascular disease plays in the development of CKD has not been explored.

A recent consensus group has been established to consider these issues further and to promote research into cardiovascular and renal disorders (CvRD).³³

Potential mechanisms by which CvRD may be detrimental to the kidney include cardiac shock, low cardiac output and hypotension resulting in reduced renal perfusion, AKI and azotemia, activation of the renin-angiotensin aldosterone system (RAAS), systemic arterial thromboembolism resulting in renal infarction and passive congestion of the kidney during congestive heart failure. In particular, inciting renal injuries that result in ischemia provide an interesting

link to the development of tubulointerstitial inflammation and fibrosis (See Chapter 3).³⁴

Systemic hypertension is a well recognized risk factor in humans for the development of CKD. Systemic hypertension is identified in approximately 20% of cats diagnosed with CKD and of cats that have evidence of systemic hypertension approximately 60% are reported to have underlying azotemic CKD at diagnosis.^{35,36} More recently, a study by Bijsmans and colleagues has demonstrated a positive association between age and increasing systolic blood pressure (SBP) and an increased risk of hypertension in those cats with CKD compared to healthy cats.³⁷ However, in longitudinal population studies, SBP has not been associated with the development of azotemia, the survival of cats with CKD or having a more progressive phenotype of CKD. The cats in these studies diagnosed with systemic hypertension however, were always treated with the calcium channel blocker, amlodipine besylate, and therefore the true effect of systemic hypertension on the development and/or progression of CKD may have been masked.³⁸⁻⁴⁰

- **Diabetes mellitus:** Unlike in human medicine, to date no association has been identified between diabetes mellitus and CKD in cats or the development of a diabetic nephropathy, although it remains possible that this reflects the relatively shorter life expectancy of diabetic cats compared to diabetic humans.^{23,41}
- **Hyperthyroidism:** Hyperthyroidism is a common condition in the older cat and therefore is often diagnosed concurrently with CKD.⁴² The effects of hyperthyroidism on renal haemodynamics are well documented from

experimental rodent studies including renal hypertrophy and an increase in GFR that is believed to be predominantly the consequence of RAAS activation secondary to change in β -adrenoceptor activity.⁴²

Hyperthyroidism has also been implicated in the progression of renal disease, the aetiopathogenesis of which is unresolved, but may relate to altered renal haemodynamics, hyperfiltration and the increased proteinuria identified in hyperthyroidism.⁴² Studies have shown that GFR declines after treatment of hyperthyroidism in cats and that, depending on the modality of therapy, between 15-49% of hyperthyroid cats will be revealed to be azotemic after treatment.^{43,44} However, this is considered to be the consequence of return to euthyroid state rather than direct renal injury. The relative contribution that being hyperthyroid makes to the development or progression of CKD in cats is unknown. Cats with hyperthyroidism have been shown to be more markedly proteinuric and that this proteinuria resolves with treatment of hyperthyroidism.⁴⁵

Proteinuria is present as a consequence of glomerular hypertension and it can be hypothesized that this in addition to protein processing by the proximal tubular cells (see below) may be detrimental to the kidney.

However, although proteinuria was significantly associated with all cause mortality in hyperthyroid cats it was not associated with development to azotemic CKD.⁴⁶ An alternative mechanism that could enable

hyperthyroidism to contribute to the development or progression of CKD is hyperparathyroidism, alterations in calcium and phosphorus homeostasis and the potential for soft tissue, including renal, mineralisation. Hyperthyroid cats have been shown to have elevated

parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) concentrations.⁴⁷ However, neither PTH or FGF23 were significantly associated with the development of azotemia in hyperthyroid cats.⁴⁷ The exact role of hyperparathyroidism and disorders of calcium and phosphorus homeostasis and their effect on renal disease in hyperthyroidism remains to be determined.

- **Upper and lower urinary tract stone disease:** Both upper and lower urinary tract stone disease are well recognized in the feline literature.^{48,49} To date there is no data specifically evaluating the effect of periods of urethral obstruction as a risk factor for the future development of CKD in cats. However, given that post-renal obstruction is an underlying aetiology for AKI it can be hypothesized that any period of urethral obstruction is likely to have a detrimental effect on the kidney. Similarly, in the past decade we have become increasingly aware in clinical practice of cats developing uretero- and nephrolithiasis of which approximately 98% of cases are related to calcium oxalate stones.⁴⁸⁻⁵⁰ It is easy to hypothesize that the acute to chronic injury caused by partial to complete ureteral obstruction, may play a role in an individual cat subsequently developing CKD. However, cats with upper urinary tract stones are typically younger (median age 7 years) than cats with CKD suggesting that this is not going to be the aetiopathogenesis for all cats with CKD but should certainly be considered in cats that present with CKD at a younger age.⁵⁰ Furthermore, a study by Ross and colleagues evaluated the presence of nephroliths as a risk factor for mortality and progression of CKD but found no association.⁵¹

- **Infectious disease:** There has been considerable interest in the potential for infectious disease to contribute to the development of CKD although evidence for these potential associations is currently poor.
 - **Urinary tract infections and pyelonephritis:** Cats with CKD are at inherent risk of urinary tract infections (UTI) due to reduced host defense mechanism. UTI are reported to affect between 17-33% of cats diagnosed with CKD, a substantial proportion of which may present as asymptomatic bacteriuria.^{3,52-54} To date only the study by Greene and colleagues has specifically evaluated prior episodes of cystitis as a risk factor for CKD in cats although it was not possible to separate the specific nature of the cystitis in this study e.g. feline lower urinary tract disease versus bacterial cystitis. Nevertheless, the presence of bacterial UTI and the possibility of ascending infections resulting in either acute or chronic pyelonephritis certainly raises concern in relation to acute or chronic kidney injury and the progression of CKD. Further work is needed to establish the significance of positive urine cultures in cats with CKD and the role that symptomatic UTI versus asymptomatic bacteriuria play in the progression of disease.⁵⁵
 - **Feline immunodeficiency virus:** Based on the presence of human immunodeficiency associated interstitial nephritis, several studies have investigated the potential role of feline immunodeficiency virus (FIV). A recent histopathological study indicated that approximately 50% of cats experimentally infected with FIV demonstrated histopathological lesions common to patients with

HIV nephritis including mesangial widening, glomerulosclerosis and immune mediated glomerulonephritis when compared to controls.⁵⁶ Histopathological lesions were more marked in kidneys from naturally affected cats that were also examined and included additional lesions such as amyloid deposits and interstitial inflammation and fibrosis.⁵⁶ However, such cats were older than the experimentally infected cats and aged matched controls were not evaluated in this study.⁵⁶ To date epidemiological studies have not been able to substantiate a clinical association between FIV and CKD.^{57,58}

- **Morbillivirus:** Several studies have raised interest in a potential association between CKD and morbillivirus infection (FmoPV), a paramyxovirus identified in cats.⁵⁹⁻⁶³ The virus was shown to have cytopathic effects in FCRK cell lines and in small numbers of stray cats there seemed to be a higher prevalence of tubulointerstitial nephritis in the cats that were positive for the feline morbillivirus than in negative cats, although little demographic and phenotypic data was available for either group.⁶² FmoPV has also been identified in the urine (10%) and blood (6%) of stray cats in a study performed in Japan, and at a higher prevalence from renal tissue (40%; 4/10) from cats with CKD.⁶⁴ The prevalence of FmoPV detected in urine by RT-PCR in client owned cats was higher at ~15%.⁶⁵ However, the prevalence of this virus in cats outside of Japan and China and any true association with the development or progression of CKD remains to be determined.

- **Leptospirosis:** Exposure of cats to leptospirosis is reported, with prevalence ranging from 3-35% depending on population and geographic location, although clinically associated disease is rarely reported.⁶⁶⁻⁶⁹ Experimental studies suggest that cats are largely resistant to acute leptospirosis although reports of clinical cases suggests that this is a possibility.^{67,70} A study by Rodriguez and colleagues has compared seropositivity and urinary PCR between CKD cats and non-age matched controls.⁶⁸ Seropositivity was significantly different between the two groups (7.2% non-CKD, 14.9% CKD cats) although PCR results were not.⁶⁸ However, a further study demonstrated no significant difference in seroprevalence between azotemic and non-azotemic cats.⁷¹ The exact role that current or prior exposure to leptospire organisms plays in feline CKD therefore requires further study.
- **Bartonellosis:** Potential associations have been made between Bartonella species infection and a number of different disease conditions but so far no association has been identified between Bartonellosis and CKD in cats.⁷²

Irrespective of the underlying aetiology and factors that may contribute to the development of CKD, it should be considered as a progressive disease. The kidney has a limited capacity to respond to an inciting injury with the main pathological findings and the end stage pathway being the development of renal inflammation and fibrosis. To date it is not completely clear whether there is a causal relationship between fibrosis and the development CKD or whether it is

purely a secondary and reparative response. Nevertheless, an understanding of the mechanisms involved in renal inflammation and fibrosis and the factors that contribute to the progression of CKD from human and experimental studies is important, as there is likely to be translational implication to the cat. If we are not able to prevent development of CKD then the greatest opportunities for intervention lie in early diagnosis (Chapters 1 and 2) and prevention of progression.

Renal inflammation and fibrosis: the kidney's response to injury

Healthy renal interstitium is composed of sparse cells (fibroblasts and dendritic cells) embedded in an extracellular matrix which is composed of collagen (I, III, VII), fibronectin and extracellular matrix glycoproteins e.g. tenascin. Renal fibrosis usually begins with focal areas of inflammation and activation of mesenchymal cells in response to an inciting injury, progressing to generate expanding areas of fibrosis and scarring.⁷³ However, after injury it can be hypothesized that fibrosis may be playing a number of different roles within the kidney. Intervention to slow or reduce fibrosis could therefore have either negative or positive effects. For example, fibrosis may occur after kidney injury when renal structural repair is incomplete with fibrosis acting as an 'innocent filler'.⁷⁴ Alternatively, fibrosis may appear after injury but interfere with the potential for full recovery. In the former scenario, preventing fibrosis would have little impact on the course of disease whereas in the latter, preventing fibrosis would be important to improve outcome.⁷⁴ Fibrosis could also be an intermediate stage, whereby the presence of fibrosis acts as a scaffold for repair. For example, studies of uranyl acetate-induced acute tubular injury suggest that

myofibroblasts may emerge after tubular damage providing a supportive structure and then regress once regeneration is complete.^{75,76} Again in this situation, prevention of fibrosis would be counterproductive. Following on from this hypothesis however, the long-term deposition of fibrosis which does not regress might be detrimental to the kidney.⁷⁴ Although fibrosis may be part of the normal healing process, in CKD fibrosis may represent a maladapted response of the kidney to injury that fails to terminate.⁷⁷ It is possible that the role of fibrosis may differ between types of renal injury. Tools that modulate fibrosis and evaluate their effect in different disease models would be required to determine whether slowing or preventing fibrosis has clinical benefit.

Renal fibrosis is characterized by an excessive fibrogenic response and expansion of extracellular matrix (ECM), which destroys the normal renal tissue. Histopathologically this is characterized by excessive accumulation of ECM, loss of renal microcirculation, infiltration of mononuclear inflammatory cells, tubular atrophy and dilation and mineralization (Figure 2). In the healthy kidney, fibroblasts play an important role in the homeostasis of ECM secretion and degradation through the production of proteases. However, during fibrosis fibroblasts become activated and transform to myofibroblasts and subsequently produce excessive quantities of ECM proteins e.g. collagen IV, laminin, fibrinectin, elastins, fibrillins, TGF- β binding proteins, tenascins and proteoglycans.

Four major cell types are important for the genesis of progressive renal fibrosis; myofibroblasts, inflammatory cells, microvascular endothelial cells (pericytes) and tubular epithelial cells.⁷⁸

- **Myofibroblasts:** The origin of myofibroblasts in the kidney has been the source of considerable controversy (Figure 3). Based on recent studies the main source accounting for over 50% of myofibroblasts is thought to be renal fibroblasts.^{79,80} Renal fibroblasts are found in the interstitium of healthy kidneys and are responsible for production of extracellular matrix and communicating with endothelial and epithelial cells. Certain sub-populations of renal fibroblasts are responsible for production of erythropoietin (EPO). In fibrosis models (e.g. unilateral ureteral obstruction (UUO) and ischaemic reperfusion (IR) injury), resident renal fibroblasts and EPO producing fibroblasts undergo a process of transformation to myofibroblasts (Figure 3).⁷⁴

Pericytes are contractile cells that wrap around microvessels and arise from mesenchymal origin.⁸¹ They play a critical role in the stability and integrity of microvessels and are able to control microcirculation by regulating capillary diameter and hence vascular tone.^{81,82} In response to injury, pericytes detach, migrate to the interstitial space and transform into myofibroblasts (Figure 2). Pericytes transformation has been demonstrated in experimental models of UUO and IR.^{83,84}

Myofibroblasts may also originate from fibrocytes (bone marrow derived myeloid precursor cells) with recent studies suggesting that fibrocytes

account for approximately 35% of myofibroblast production.^{74,80} Early studies suggested that local renal cells including tubular epithelium might also undergo either epithelial mesenchymal transition (EMT) and contribute to the myofibroblast population.^{85,86} However, conflicting studies have been published using in-vivo data and EMT is likely to represent only a very small (<5%) component of myofibroblast production.⁸⁰ Endothelial cells are also capable of endothelial-mesenchymal transition with a recent study suggestion they account for approximately 10% of myofibroblast production (Figure 3).^{87,88}

Myofibroblasts have the characteristics of both fibroblasts and smooth muscle cells. Key features include expression of alpha smooth muscle actin (α -SMA) and vimentin (intermediate filament protein), abundant rough endoplasmic reticulum and their ability to secrete pericellular matrix containing collagen and glycosaminoglycans. The recruitment and transdifferentiation of precursors to myofibroblasts can be activated by both local and circulating factors. Important stimuli include autocrine and paracrine growth factors (e.g. TGF- β , platelet derived growth factor (PDGF), VEGF, connective tissue growth factor (CTGF) of which perhaps the most important is TGF- β . TGF- β production can be stimulated by many factors recognized to promote renal injury including RAAS, proteinuria, increased single nephron GFR (SNGFR) and oxidative stress.⁸⁹⁻⁹³ Other stimuli for the activation of myofibroblasts include direct interaction with leukocytes/macrophages (see below) and tubular epithelial cells (see below) and local environmental stimuli such as hypoxia and hyperglycemia.

- **Inflammatory cells:** Inflammatory cells, particularly macrophages, play an important role in inflammation, tissue repair and fibrosis. Sub-populations of macrophages may be responsible for production of inflammatory mediators, for example IL1 β , TNF- α , chemokines (Monocyte Chemoattractant Protein-1; MCP1) which recruit further inflammatory cells, increase production of TGF- β and PDGF which promote myofibroblast activation.⁷⁷ It has also been speculated that monocytes from the circulation may enter renal tissue and differentiate into fibrocytes (Figure 2). However, the evidence base for this is largely based on cell culture systems.^{74,94,95}
- **Pericytes:** The role of pericytes differentiating to form myofibroblasts has been described above. However, pericytes are also important for vascular stability. The loss of pericytes, as they transform to myofibroblasts, contributes to loss of the interstitial capillary network, otherwise known as peritubular capillary rarefaction and may play a major role in the development of hypoxia, oxidative stress and progressive tubular cellular injury and fibrosis.^{77,78,81,96} Histological studies evaluating human kidney tissue have demonstrated that areas of renal fibrosis and reduced peri-tubular capillary density located together.^{97,98} Hypoxia is a recognized final common pathway for the progression of CKD (see below).^{99,100} In addition, increase in permeability of microvasculature and leakage of plasma proteins such as fibrinogen and albumin into the interstitium may trigger an inflammatory response.¹⁰¹

- **Tubular epithelial cells:** Tubular epithelial cells have a number of roles in renal fibrosis. Early in the course of the disease injured tubular epithelial cells may be a source of inflammatory mediators, e.g. cytokines and chemokines (IL6, MCP-1, tumor necrosis factor- α) which recruit and activate inflammatory cells and promote differentiation of fibroblasts to myofibroblasts.¹⁰² A study by Yang and colleagues demonstrated that tubular epithelial cell cycle arrest in response to toxic, obstructive and ischemic injury resulted in the development of fibrosis.¹⁰³ In response to injury, tubular cells may also increase production of growth factors (e.g. TGF- β , PDGF, fibroblast growth factors (FGF)) and reactive oxygen species (ROS). When secreted either via the paracellular route or to the basolateral membrane into the tubulointerstitium these factors all have pro-fibrogenic effects.^{74,78} Urinary complement factors and cytokines and protein handling by the tubular cells have also been implicated (see below) as stimuli for the release of pro-inflammatory and pro-fibrotic mediators from tubular cells.^{78,104} Later in the disease process, lack of regeneration and loss of tubular epithelial cells may contribute to progression of disease and loss of nephrons.

Evidence of fibrosis in feline chronic kidney disease:

The main histopathological finding reported in older cats with CKD is tubulointerstitial inflammation and fibrosis.^{3,4} In human medicine, even in patients with primary glomerular disease, it is tubulointerstitial lesions that correlate most strongly with renal function. Recently feline studies have compared histopathological findings with stage of kidney disease and identified

that the severity of tubular degeneration, interstitial inflammation, fibrosis and glomerulosclerosis was more marked in the later IRIS stages of CKD.² In a study by Chakrabarti and colleagues, fibrosis was the only histopathological finding that significantly correlated with severity of azotemia and IRIS stage.¹ To date two studies have specifically evaluated myofibroblast recruitment in cats with CKD.^{105,106} The presence of myofibroblasts in feline renal tissue, identified by the markers α SMA and tubular and interstitial vimentin, correlated positively with both fibrosis and plasma creatinine concentration.¹⁰⁶ Expression of α SMA and fibronectin were significantly higher in cats with tubulointerstitial nephritis particularly in the peri-glomerular and peri-tubular areas and were associated with severity of azotemia.¹⁰⁵ It was also evident that α SMA was expressed at earlier stages of tubulointerstitial nephritis and in some apparently prior to the deposition of ECM.¹⁰⁵

A limited number of feline studies have also evaluated some of the inflammatory markers that may be stimuli for fibrosis and inflammation including TGF- β .

Preliminary studies support increased urinary TGF- β in cats with CKD compared to controls and a positive correlation between urinary TGF- β and azotemia.^{107,108} However, conflicting studies evaluating active rather than total urinary TGF- β 1 found no significant difference between non-azotemic geriatric cats, non-azotemic cats that progressed to develop azotemia and azotemic cats. However, there was a trend towards increasing active urinary TGF- β in cats that developed azotemia and were monitored longitudinally.¹⁰⁹ A study by Habenicht and colleagues investigated urinary concentrations of IL8 and MCP-1 as ratios to urine creatinine as potential markers of renal inflammation and injury. No significant difference in MCP-1:creatinine ratio could be detected although IL8:

creatinine ratio were significantly higher in cats with CKD than control cats.¹⁰⁷ A shortfall of these studies is that histopathology was not routinely available although in other species studies support that the correlation between urinary cytokine levels and renal inflammation is strong.¹¹⁰

Transglutaminase 2 (TG-2) has recently been investigated in cats. TG-2 is a calcium dependent cross-linking enzyme from the transglutaminase family, which plays an important role in stabilizing extracellular matrix thereby promoting extracellular matrix deposition and resistance to degradation. There is a strong relationship between TG-2 expression and renal fibrosis in humans with CKD and also rodent models.^{111,112} This has recently been extrapolated to the cat where TG-2 activity was positively correlated with renal histopathology scoring, plasma creatinine, phosphate and urea concentrations.¹¹³

Together these findings begin to support the importance of fibrosis in the pathogenesis of CKD although the further work is required to determine whether fibrosis is detrimental and whether modulating fibrotic pathways holds any clinical benefit.

Mechanisms of progression and maladaptive repair in chronic kidney disease:

In clinical practice, many cats with CKD remain stable for many years. Median survival time of cats with azotemic IRIS stage 2, 3 and 4 CKD have been reported as 1151, 778 and 103 days respectively although there is considerable inter-individual variability and survival times reported are potentially affected by the modalities of therapy, interventions and resources available.^{24,38} However, ultimately some cats do demonstrate progressive disease although the time

point at which that progression occurs is often unpredictable.¹¹⁴ In one study 29% of cats with IRIS stage 2 disease and 63% of IRIS stage 3 CKD cats progressed to IRIS stage 4 before death.⁴⁰ Epidemiological studies have evaluated factors associated with the development of azotemia, survival of cats with CKD and also demonstrating a more progressive phenotype of CKD.^{24,38-40,115-117} From the experimental and human literature there are a number of key pathophysiological mechanisms which have been implicated in both the development and progression of CKD. An understanding of these mechanisms is important not only because many may be applicable and translated to feline CKD but also because they provide potential targets for therapy in order to slow disease progression.

Hemodynamic adaptations:

In the 1980's, experimental rodent studies were performed that indicated that there was a haemoadaptive response that occurred as a consequence of nephron loss and which resulted in glomerular hypertrophy, hypertension and hyperfiltration. The net effect of these haemodynamic alterations was maintaining and increasing single nephron GFR (SNGFR) in order to preserve renal function. However, although initially beneficial in terms of maintaining total GFR, ultimately these adaptations were detrimental and a critical point would be reached where self-perpetuating loss of further nephrons would result in progression of disease.

There is evidence from experimental feline studies that similar haemodynamic and structural adaptations occur in cats. Early feline renal mass reduction studies documented histopathological evidence of glomerular lesions, fibrosis

and mineralization and yet when followed over a year, these cats did not demonstrate the continued progressive decline in GFR that would be anticipated from the rodent studies. Micropuncture studies in cats showed that in response to renal mass reduction there was evidence of dilation of pre-glomerular afferent arterioles, increase in glomerular capillary pressure and as a consequence increased effective filtration pressure and SNGFR. Glomerular hypertrophy with a secondary increase in ultrafiltration coefficient, mesangial matrix expansion and increased proteinuria were also observed. Cats were therefore documented to show similar haemoadaptive and structural changes to those occurring in rodent models although the changes were, to some extent, dependent on the degree of renal mass reduction. Thus, it has been hypothesized that the predisposition of an individual to progressive CKD may be a balance between adaptive mechanisms preserving GFR versus the development of structural lesions that advance disease.

The role that the haemoadaptive and structural glomerular changes play in naturally occurring feline CKD is more difficult to quantify. Studies evaluating renal pathology at post-mortem examination have scored glomerular lesions included glomerular hypertrophy, glomerulosclerosis and obsolescence (global matrix expansion with loss of capillary lumina). Stage 2 CKD cats demonstrated significantly increased glomerular volume compared to non-azotemic control cats whereas in stage 3 and 4 CKD cats glomerular volume was closer to the control population. It was hypothesized that this could be explained if glomerular hypertrophy occurring earlier in the course of CKD as an adaptive

process, subsequently became maladaptive resulting in glomerulosclerosis and obsolescence.

Activation of the renin-angiotensin-aldosterone system:

It is widely accepted that the RAAS is activated in CKD and that both systemic and tissue specific RAAS systems exist. The kidney contains all the necessary components for local RAAS activation and indeed renal tissue concentrations of angiotensin II (Ang II) are reported to be significantly higher than plasma concentrations.^{118,119} As such, quantification of plasma components of RAAS does not necessarily translate to the degree of activation of RAAS within the kidney.¹²⁰ RAAS is particularly important as a modulator of blood pressure and fluid balance through alteration in sodium and water homeostasis as well as being integral to intrarenal haemodynamics and glomerular filtration. Traditionally, the RAAS pathway terminates with conversion of Ang I by angiotensin converting enzyme (ACE) or alternative pathways (e.g. chymase) to Ang II. Ang II mediates its effects predominantly via the type 1 (AT1) receptor which has a wide distribution throughout the kidney although type 2 receptors (AT2) are present albeit with a more limited distribution.¹¹⁸ However, it is now recognized that a number of further products of both Ang I and Ang II may play important regulatory roles (e.g. Ang (1-7)/Mas and Ang IV/AT₄ pathways) and may have impact in the pathogenesis of CKD (Figure 4).^{118,121,122}

Ang II in particular has been highlighted as an important mediator in the progression of CKD. It acts as a potent vasoconstrictor contributing to the development of glomerular hypertension and hyperfiltration and that it may

modulate the permeability of the glomerular filtration barrier at least partly by altering podocyte interactions.¹²³⁻¹²⁵ Therefore Ang II may not only promote renal injury through sustained glomerular hypertension and development of glomerulosclerosis but also by promoting proteinuria which itself may be detrimental to the kidney (Figure 4; see below).¹²⁶ Ang II also has direct fibroproliferative and inflammatory effects by increasing transcription and production of inflammatory and profibrogenic molecules including TGF β hence promoting myofibroblast transformation and fibrosis (Figure 4).⁹² Ang II may play a role in the migration and transformation of pericytes to myofibroblasts and also the differentiation of circulating fibrocytes to fibroblasts. It has been reported to stimulate the production of other growth factors and inflammatory mediators from vascular smooth muscle cells, glomerular endothelium and mesangial cells (e.g. MCP-1 and RANTES) and is a known activator of NF κ B which is a key transcription factor in inflammatory disease stimulating up-regulation of genes encoding for pro-inflammatory cytokines.^{92,127,128}

The vasoconstrictive effects of Ang II on the efferent arteriole result in structural and functional changes to the peritubular microvasculature (Figure 4). Reduced renal oxygenation due to the effects of vasoconstriction may manifest before the capillary rarefaction which is identified as part of renal fibrosis.^{96,129} In medullary interstitial cells, Ang II has been shown to activate hypoxia inducible factor (HIF-1 α) via reactive oxygen species (ROS) generation and *in vivo* medullary interstitial cells from kidneys perfused with Ang II stain positively for both HIF-1 α and α SMA. These findings together link Ang II to both hypoxia and oxidative stress mediated renal fibrosis mechanisms.¹³⁰

Production of aldosterone by the adrenal gland is stimulated by Ang II but may itself have pro-fibrotic effects contributing to the pathogenesis of CKD.¹³¹ Local renal aldosterone production in the renal cortex has been demonstrated in rodent models stimulated by Ang II, decreased sodium intake and hyperglycemia with the mineralocorticoid receptor identified not only in the distal tubule but also in pre-glomerular vasculature, mesangial cells and on fibroblasts.¹³² *In vitro* studies have shown that mesangial cells significantly increase production of TGF β and fibronectin in response to aldosterone, an effect which can be mitigated by the aldosterone antagonist, spironolactone.¹³³ *In vivo* aldosterone infusion in rats significantly increases urinary TGF β concentration and rats that have undergone uninephrectomy and receive aldosterone at the same time as AT1 blockade, showed increased expression of TGF β and collagen, supporting that aldosterone is an independent pro-fibrotic mediator.^{132,134,135} Aldosterone has also been shown to increase expression of CTGF and to increase production of ROS and inflammatory mediators such as osteopontin, IL6 and IL1.^{136,137} Aldosterone may also promote fibroblast growth and proliferation and increase expression of PAI-1, which promotes ECM accumulation.^{131,132} Numerous *in vivo* models of aldosterone inhibition using either the non-selective agent, spironolactone, or the specific aldosterone receptor inhibitor, eplerenone, demonstrate reduction in glomerulosclerosis and interstitial fibrosis.¹³²

In human medicine, clinical evidence of the role of RAAS in progression of CKD comes from studies that have evaluated RAAS blockade. Studies investigating both ACE inhibitors (ACEi) and angiotensin receptor blockers (ARB) show

improved outcome when these agents are administered for both diabetic and non-diabetic related CKD.¹³⁸ However, outcomes from these studies have not provided the degree of protection that might be anticipated and more recent studies have therefore focused on combined ACEi and ARB therapy.^{138,139} Whilst dual therapy may provide further reduction in terms of proteinuria outcome measures such as requirement for dialysis, doubling of serum creatinine and mortality were not always improved when compared to monotherapy particularly for non-proteinuric patients.¹⁴⁰ There continues therefore to be controversy in terms of optimal therapy for RAAS blockade and whether this should be monotherapy, combined fixed dose or individualized ACEi/ARB or combined therapy with either aldosterone antagonists or direct renin inhibitors (e.g. Aliskiren).^{141,142}

There is relatively little data in the literature evaluating RAAS activation in cats with CKD. In experimental feline models of renal mass reduction, significant increases in plasma renin activity (PRA) and aldosterone have been documented.¹⁴³⁻¹⁴⁵ In cats that underwent a renal wrap model of renal reduction, increased plasma renin, plasma aldosterone, systemic hypertension, proteinuria and more marked histopathological changes were reported.¹⁴⁴ These findings supported RAAS activation in association with this feline experimental model of renal mass reduction although this may not be directly translated to naturally occurring disease.

A small number of studies have investigated intra-renal RAAS in cats with naturally occurring CKD by immunohistochemistry.¹⁴⁶⁻¹⁴⁸ The first study by

Mitani and colleagues evaluated expression of renin and Ang II in feline kidneys. Renin was identified in the afferent arteries and Ang II in the proximal tubules and mononuclear cells. No association was identified between immunostaining of renin with severity of azotemia or histopathological lesions but tubular and interstitial Ang II immunostaining correlated with glomerulosclerosis and tubulointerstitial inflammation.¹⁴⁷ The second study by Mitani and colleagues evaluated ACE and ACE2 (mediates production of Ang1-7/Mas pathway) expression. ACE was identified predominantly in the proximal tubules whilst ACE2 was identified in proximal tubules and weaker staining in the distal nephron. Unlike in dogs, there was no association between immunostaining of ACE or ACE2 with histopathology scores although sample numbers were small.¹⁴⁸ Further work and larger studies are required to confirm the association between altered expression of these components of RAAS with severity and progression of CKD.

Several studies have evaluated plasma components of RAAS in cats with naturally occurring CKD, some of which were also hypertensive although results have been variable.¹⁴⁹⁻¹⁵¹ A study investigating PRA and aldosterone concentration in normotensive azotemic CKD and non-azotemic age matched controls found no significant difference between these variables.¹⁵⁰

Experimental feline studies have demonstrated that administration of ACEi alters renal haemodynamics and reduces proteinuria with the latter observation also been observed in cats with naturally occurring CKD.^{143,152-154} However, to date, the survival advantage that we might anticipate from administration of an ACEi has not been demonstrated.¹⁵² Clinical equivalency in terms of anti-

proteinuric effect has been documented between the ARB, telmisartan and ACEi but results of on-going studies to evaluate the effect of this alternative approach on RAAS inhibition and progression of renal disease, renal associated mortality and survival are required.¹⁵⁵ Overall, given the expanding evidence base from *in vitro* and *in vivo* experimental studies and from human medicine it seems likely that RAAS is an important player in the pathogenesis of feline CKD. However, the optimal way to inhibit RAAS to improve outcome remains to be determined.

Systemic hypertension

Systemic hypertension has been accepted for many decades in human medicine as both a factor implicated in the development and the progression of kidney disease.¹⁵⁶⁻¹⁵⁸ Early experimental rodent models demonstrated that with reduced renal function, pre-glomerular vasodilation occurs, permitting over-ride of myogenic renal autoregulation, transfer of elevated systemic pressures to the glomerular capillaries resulting in glomerular hypertension and glomerulosclerosis.¹⁵⁹ Histopathological lesions associated with hypertension in the kidney include arterosclerosis (arterial intimal thickening, medial hypertrophy and duplication of the internal elastic lamina) glomerulosclerosis and tubular atrophy which, together in human medicine has been termed arterionephrosclerosis. Systemic hypertension and glomerular hypertension has also been associated with more marked proteinuria both in experimental rodent studies and also in human studies.^{159,160}

The evidence for systemic hypertension contributing to the development or progression of feline CKD is unclear. In feline experimental renal mass reduction

models, systemic hypertension has been associated with histopathological changes.¹⁴⁴ However, epidemiological studies evaluating factors associated with the development of azotemia, the survival of cats with CKD and having a more progressive phenotype of CKD have not identified blood pressure as a risk factor.³⁸⁻⁴⁰ A conflicting factor in all of these studies however, is that cats diagnosed with systemic hypertension received anti-hypertensive therapy and therefore it is not possible to say whether an association would have been identified had untreated cats been included. Cats with systemic hypertension are more proteinuric than normotensive cats with equivalent stage of CKD.³⁸ A study evaluating factors associated with the survival of cats with systemic hypertension evaluated time-averaged blood pressure as a variable giving information about degree of control of SBP despite all cats receiving anti-hypertensive therapy. When cats were divided into quartiles based on their time-averaged SBP those in the upper quartile would still have been considered hypertensive despite anti-hypertensive therapy. Never-the-less, only proteinuria was significantly associated with survival.³⁶ This raises questions regarding the role that systemic hypertension plays in the progression of CKD in cats and whether proteinuria might not be the more important factor.

A histopathological study performed by McLeland and colleagues demonstrated no difference in severity of vascular lesions (vascular hyperplasia, arteriosclerosis, glomerulosclerosis) between hypertensive and normotensive cats with naturally occurring CKD.² However, not all cats in this study had blood pressure data available and only 12 cats were diagnosed ante-mortem with systemic hypertension. A further study by Chakrabarti and colleagues with blood

pressure data on 69 cats of which 34 had a diagnosis of systemic hypertension also evaluated post-mortem renal pathology.¹ The most common hypertension associated lesion identified was hyperplastic arteriosclerosis in 3% of normotensive and 29% of hypertensive cats and in a multivariable model, mean glomerular score and hyperplastic arteriosclerosis were significantly and independently associated with time averaged blood pressure.¹ As for previous studies all cats in this study diagnosed with systemic hypertension received anti-hypertensive therapy with amlodipine besylate. Nevertheless, the median time averaged blood pressure of those cats diagnosed with systemic hypertension that demonstrated hyperplastic arteriosclerosis was 171mmHg versus 152mmHg for those without.¹ There was however no association between time-averaged blood pressure and tubulointerstitial inflammation or fibrosis and it is difficult to fully interpret the relative effect of blood pressure on these histopathological changes versus the effect that administration of amlodipine besylate, and afferent arteriolar vasodilation in the face of inadequate blood pressure control may have played.

Given the associations that have been made in experimental models of hypertension it is reasonable to perceive that the kidney should be considered a target organ of systemic hypertension and that appropriate anti-hypertensive therapy should be administered accordingly. The role that systemic hypertension plays in the progression of CKD remains to be fully determined but will prove challenging to evaluate in clinical patients where withholding anti-hypertensive therapy is not ethically justified. Further studies are also warranted to further investigate the role of proteinuria in hypertensive CKD

patients and to determine whether blood pressure alone or in combination with proteinuria would be a better end target.

Proteinuria

Proteinuria as a consequence of glomerular hypertension, hyperfiltration and change in permselectivity of the glomerular filtration barrier is proposed to promote an apoptotic response in tubular cells, alter phenotype of tubular cells and contribute to the development of tubulointerstitial inflammation and fibrosis.¹⁶¹ In health, filtered proteins are reabsorbed by megalin and cubulin mediated endocytosis in the proximal tubules such that the magnitude of proteinuria is low.¹⁶² However, the process of protein presentation and reabsorption by the proximal tubular cells (PTC) is not considered benign.¹⁶¹

For over a decade, there has been evidence from *in vitro* studies, that proteins such as albumin (delipidated or lipidated), immunoglobulin G, and transferrin presented to the apical surface of PTC grown in monolayers, upregulate the gene expression and production of vasoactive (e.g. endothelin-1), pro-inflammatory (MCP-1, RANTES, IL8) and profibrotic factors (e.g. TGF β). Important intermediate mediators have been identified to include NF κ β and ROS and megalin has been implicated as a central element linking protein absorption and the intracellular pathways that up-regulate gene expression.^{104,161} Basolateral release of these modulators raises the potential that *in vivo* release would potentiate tubulointerstitial inflammation, fibrosis, ECM deposition and progression of CKD.

There has been debate regarding exactly which protein molecules are most potent in terms of stimulating the inflammatory response and also whether it is protein alone or in association with their fatty-acid binding capabilities (e.g. oleic and linoleic acid) that is most important in stimulating this response.

Controversy also exists as to whether the concentrations of proteins used in these *in vitro* studies corresponds to or exceeds the protein concentration of ultrafiltrate *in vivo* and therefore whether results are directly applicability particularly to patients with primary tubular disease. Rodent models of proteinuria were used to substantiate the role of proteinuria as a stimulus for an inflammatory and pro-fibrotic response demonstrating up-regulation of many of the inflammatory mediators, e.g. MCP-1, osteopontin, NF κ β , and that these changes lead to inflammatory cell recruitment to the tubulointerstitial space.^{163,164} These changes could be abrogated by administration of anti-proteinuric therapy e.g. ACEi.^{165,166}

Complement activation is a powerful mechanism that can promote both pro-inflammatory and profibrotic effects either via the classical or alternative pathway in the kidney. There is evidence that both intra-renal and filtered C3, an essential factor of both the classical and alternative pathways of complement activation, may promote formation and insertion of the C5b-9 membrane attack complex.^{167,168} Studies in proteinuric rodent models have shown that C3 colocalises to the apical surface of proximal tubular cells in advance of the recruitment of inflammatory cells and that these changes can be mitigated by administration of ACEi.¹⁶⁹

However, other perhaps less well-documented effects of proteins on the PTC have been reported. A study by Cao and colleagues indicated that high concentrations of albumin presented to PTC stimulated activation of renal RAAS.¹⁷⁰ Studies support that accumulation of non-esterified fatty acids (NEFA) and long chain acyl-co A transported into the tubule bound to albumin may accumulate in PTC and be a stimulus for PTC apoptosis.¹⁶¹ *In vitro* studies using cell culture of both human and rodent PTC have shown that albumin can directly lead to cellular apoptosis via a caspase-9-mediated mitochondrial pathway and that megalin may be an important receptor for this pathway.^{171,172} *In vivo*, proteinuria has been associated with tubular atrophy and number of apoptotic cells but such studies have primarily been performed in disease conditions or models of marked proteinuria (e.g. Heymann nephritis and focal segmental glomerulosclerosis).^{173,174} Further work is therefore required to determine the significance of this mechanism in primary tubular disease conditions. In human medicine numerous studies of both diabetic and non-diabetic CKD have demonstrated that proteinuria is associated with faster GFR decline and progression to end stage renal disease with evidence that anti-proteinuric therapy with an ACEi or ARB can slow this decline although as described above dual therapy does not always enhance outcome.^{138,140}

For cats where the primary histopathological lesion is tubulointerstitial nephritis, the magnitude of proteinuria is typically low with reported median urine protein to creatinine ratios in IRIS stage 2, 3 and 4 CKD being 0.15, 0.22 and 0.65 respectively.³⁸ In feline CKD, proteinuria has been significantly associated with the development of azotemia, having a progressive phenotype of

CKD, and survival of cats with both CKD and hypertension.^{36,38-40} In histopathological studies of feline kidney tissue at post-mortem, proteinuria has been significantly associated with the severity of tubular degeneration, inflammation, fibrosis, tubular epithelial cell necrosis and decreased amount of normal renal parenchyma.^{1,2} However, although both ACEi and ARB significantly reduce magnitude of proteinuria in cats, the benefit in terms of slowing progression of CKD or improving survival has yet to be demonstrated.^{152,155} On the basis of data from experimental studies proteinuria is a key player in the pathogenesis of interstitial inflammation and fibrosis. However, further work is still required to demonstrate in feline medicine the causative association between proteinuria and progression of disease.

Hyperphosphatemia

Hyperphosphatemia as a consequence of decreased renal excretion has been associated with progression of renal disease in human studies.¹⁷⁵ Early rodent studies showed that diets with excessive phosphorus supplementation predisposed to histopathological lesions within the kidney including necrosis of the convoluted tubules, calcification and an increase in ECM.^{176,177} Conversely, experimental models of renal disease including the dog have shown that phosphate restriction is beneficial in reducing renal injury.^{178,179} The proposed pathogenesis for phosphate being detrimental to the kidney is incompletely understood and a number of key mechanisms have been proposed. The most frequently cited is that hyperphosphatemia predisposes to renal mineralization, which subsequently promotes inflammation and fibrosis. Alternative proposed mechanisms include an association between hyperphosphataemia and vascular

calcification leading to vascular stiffness with subsequent effects on endothelial cell function.¹⁸⁰⁻¹⁸³ These alterations in renal microvascular may be contributory to ischemia and hypoxia, which are known stimuli for renal fibrosis (see below).¹⁸³ Phosphate may also affect a number of other key pathways that have been associated with renal disease progression including cellular apoptosis, cellular senescence and oxidative stress.^{18,184,185} Extracellular phosphate concentrations have also been associated with increased production of profibrotic mediators and may have a direct stimulatory effect on RAAS.¹⁸⁶⁻¹⁸⁸

There is some evidence supporting the role of phosphorus in the pathogenesis of feline CKD. Early studies evaluating phosphate restricted diets showed that cats on restricted diets had reduced evidence of renal histopathological lesions (calcification, fibrosis and inflammatory cell infiltration) compared to cats on a high phosphorus diet.¹⁸⁹ Clinical epidemiological studies have identified phosphorus to be a risk factor for the survival of cats with CKD and also to be associated with a more progressive phenotype of disease.^{24,40} To date one feline study has evaluated post-mortem renal histopathological findings with pre-mortem phosphate concentrations and identified a significant association with interstitial fibrosis but not with renal mineralization.¹ More recently epidemiological studies have focused on the role of other molecules involved in phosphorus homeostasis with both PTH and FGF23 being associated with the development and survival of cats with CKD.¹¹⁵⁻¹¹⁷ Perhaps the most convincing evidence that there is likely to be a role for phosphorus homeostasis in the progression of CKD comes from the studies that have demonstrated improved

survival in those cats that are fed a phosphate restricted diet with evidence of modulation of these important regulatory hormones.^{114,190,191}

Hypoxia

Hypoxia has been associated with the development and progression of CKD. A number of different potential mechanisms may contribute to hypoxia.¹⁹² The total blood supply to the kidney is high, representing approximately 20% of cardiac output. However, the presence of the counter-current multiplier system within the kidney and oxygen diffusion shunt means that the renal medulla operates at low oxygen tensions. The high metabolic demand of proximal tubular cells means that they may be particularly susceptible to reduced availability of oxygen, a mechanism that is used for beneficial effect as a driving factor for the production of erythropoietin.

In the kidney, afferent arterioles divide to give rise to glomerular capillaries, which fuse to become the efferent arteriole. Efferent arterioles enter the peritubular complex of capillaries, and provide oxygen and nutrients to the tubular cells. Early in the course of CKD alteration in glomerular structure and the development of glomerulosclerosis may alter the delivery of blood from the glomerulus to peri-tubular capillaries, limiting the blood supply to renal tissue (Figure 5). Haemodynamic alterations, driven at least in part by activation of the RAAS (see above) and Ang II, result in relative vasoconstriction of the efferent arterioles.¹²⁹ The benefit of this may be increase in glomerular capillary pressure and filtration fraction, but this haemodynamic alteration has secondary consequences on the supply of blood and hence oxygen from the efferent arteriole to the peritubular capillary network and may result in hypoxia.¹²⁹

Delivery of oxygen to tubular cells is dependent on diffusion. In health, when there is little distance between the peritubular capillaries and tubular epithelial cells, oxygen is delivered efficiently. However, once there is evidence of tubulointerstitial inflammation and fibrosis, the distance for oxygen molecules to traverse increases, contributing to hypoxia. Furthermore, loss of interstitial microvascular and capillary rarefaction may reduce blood supply to regions of the kidney.^{99,192}

As part of the normal physiological response to hypoxia, cells undergo adaptations in gene expression in order to try to counteract the effect of low oxygen. This response typically involves stimulation of hypoxia inducible factors (HIF)-1 and HIF-2.¹⁹³ In the kidney, the primary form of HIF expressed in the tubules and interstitial cells is HIF-1 α whereas HIF-2 α is identified in mesangial cells, endothelial cells and fibroblasts. The HIF α -subunits are degraded by an oxygen dependent mechanism. This means that in periods of hypoxia HIF- α subunits accumulate and form heterodimers with HIF- β subunits. These heterodimers are able to bind to hypoxia response elements associated with key genes that counteract the effects of hypoxia e.g. erythropoietin, vascular endothelial growth factor (VEGF) which stimulates angiogenesis, heme oxygenase which is involved in heme metabolism, nitric oxide synthase and cyclo-oxygenase-2 which both have vasodilator properties.^{193,194,195}

Hypoxia itself has also been shown to be a fibrogenic stimulus to tubular epithelial cells, fibrocytes and endothelial cells.¹⁹⁶ Hypoxia has been shown to

stimulate tubular epithelial cells to undergo epithelial to mesenchymal transition and activate fibroblasts to increase production of ECM.^{197,198} Some tubular epithelial cells that are exposed to hypoxia develop mitochondrial derangements that result ultimately in cellular apoptosis. A secondary consequence of loss of normal renal parenchyma is a decrease in the availability of fibroblasts for the production of erythropoietin, which later in the disease course of CKD may result in anaemia. Anaemia in CKD may therefore also contribute to reduced oxygen delivery and hypoxia. Overall, therefore the haemodynamic and structural lesions that occur in CKD, contribute and promote hypoxia which is in itself is a driving factor contributing to renal fibrosis and generating a vicious cycle for disease progression (Figure 5).¹⁹²

In cats there is relatively little information directly relating to hypoxia and its association with the development and progression of CKD. Epidemiological studies have identified packed cell volume and anaemia as being risk factors for the survival of cats with CKD and anaemia is clinically appreciated to contribute to reduction in quality of life.^{24,199} In a more recent study, low packed cell volume was an independent predictor of progression of CKD in cats with IRIS stage 2 CKD.⁴⁰ Together these studies give us preliminary evidence that anaemia, which could certainly contribute to renal hypoxia, is a negative prognostic indicator in terms of progression of disease and survival.

More recently studies have evaluated urinary VEGF: creatinine concentrations as a method of assessing response to hypoxia within the kidney. Results from these studies so far have been conflicting. A study by Habenicht and colleagues

demonstrated significantly lower urinary VEGF:creatinine ratio in cats with CKD compared to healthy controls.²⁰⁰ This could be hypothesized to suggest inadequate response of the kidney to the hypoxic environment. Further work is necessary to clarify the extent to which plasma VEGF concentrations might contribute to urinary VEGF concentrations and to establish further robust markers that may be informative in terms of the role of hypoxia in the progression of CKD in cats.

Oxidative stress

Renal oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS; e.g. superoxide, hydroxyl radical and hydrogen peroxide)) and availability of anti-oxidant defense mechanisms (e.g. superoxide dismutase, catalase, glutathione peroxidase, and glutathione). ROS are highly reactive molecules, which can cause damage to DNA, lipid, protein, carbohydrate resulting ultimately in structural and functional cellular damage that leads to apoptosis and necrosis, stimulating inflammation and fibrosis. This imbalance is a situation that is referred to as oxidative stress.²⁰¹

Renal cells, particularly tubular cells, have a high metabolic activity and therefore have a high production rate of ROS. The major site of production of ROS is the mitochondria although endoplasmic reticulum, peroxisome and lysosomes may also contribute.²⁰² Oxidative stress occurs in CKD when hyperfiltration and hyperfunctioning of remaining nephrons leads to increased production of ROS which influence downstream cellular signaling pathways in the kidney to promote renal cell apoptosis, cellular senescence, a decrease in the

regenerative capacity of the cells and fibrosis.^{89,203} In particular ROS have been shown to stimulate NFκβ which is integral in cellular pathways that promote fibrosis. These factors have deleterious effects in terms of both renal disease progression and reducing renal function. Other factors such as aging, proteinuria, RAAS activation and angiotensin II, hyperphosphataemia, inflammation, regions of ischaemia and hypoxia, and uremic toxins can also contribute to the generation of ROS.^{201,204}

Assessment of oxidative stress in clinical patients is challenging and evaluation of biomarkers for oxidative stress is often used in place of direct assessment. These biomarkers may be informative in terms of lipid peroxidation (e.g. Isoprostanes, malondialdehyde, thiobarbituric acid reactive substances), protein oxidation (e.g. protein carbonyls, advanced glycation end products, oxidized low-density lipoproteins), direct measurement of ROS (e.g. hydrogen peroxide, DNA or RNA damage) or may be through evaluation of antioxidant mechanisms (e.g. catalase, glutathione, reduced-oxidized glutathione (GSH:GSSH), glutathione peroxidase, superoxide dismutase).²⁰¹ In human medicine, studies have shown increase in oxidative stress markers with advancing stage of CKD.^{205,206} However, the response to provision of antioxidants (e.g. α-tocopherol, ω-3 fatty acids, n-acetyl cysteine, allopurinol, co-enzyme Q₁₀) has been variable and a recent Cochrane review found no evidence that anti-oxidant therapy could reduce death or cardiovascular disease in human patients with CKD.²⁰⁶ The study did suggest that anti-oxidant therapy may lead to reduced serum creatinine and may therefore reduce the risk of progression to end stage renal disease and there was no evidence that supplementing with anti-oxidants was harmful. However, the

review was based on few studies and overall a low event rate. The conclusion of this Cochrane review was therefore insufficient evidence to support routine use of anti-oxidant therapy at this time although there was sufficient evidence to support that further studies should be performed to explore the potentially beneficial effects of anti-oxidant therapy.²⁰⁶

There have been few studies to date that have explored oxidative stress in relation to feline CKD.²⁰⁷ Two studies have attempted to evaluate oxidative stress in cats with CKD using different methods. A study by Keegan and colleagues, evaluated superoxide dismutase, antioxidant capacity, GSH:GSSG ratio, neutrophil phagocytosis and oxidative burst in cats with CKD and age matched control cats. Results from this study indicated that cats with CKD had significantly higher GSH:GSSG ratios and significantly reduced antioxidant capacity.²⁰⁸ There was no significant difference in superoxide dismutase activity between groups whilst neutrophil burst was significantly higher in the CKD cats.²⁰⁸ Together these results were interpreted as evidence that anti-oxidant mechanisms are activated in cats with CKD. Krofic Zel and colleagues measured selenium concentrations, plasma and erythrocyte glutathione peroxidase activity and total plasma antioxidant capacity. Selenium concentrations were investigated because selenium is an integral component of glutathione peroxidase. This study identified that IRIS stage 4 cats had significantly higher plasma glutathione peroxidase activity but that there was no significant difference in the other markers either amongst IRIS stage or between CKD and control cats.²⁰⁹ These results suggest that at stage 4 CKD cats may still be able to induce

anti-oxidant mechanism and that selenium deficiency does not seem to be a factor in cats with IRIS stage 1-4 CKD and a non-age matched control group.²⁰⁹ To date only one study has evaluated anti-oxidant supplementation in cats. This study evaluated dietary supplementation with vitamin C and E in a group of 10 elderly cats with CKD compared to healthy non-age matched controls.²¹⁰ They used 8-hydroxy-2'-deoxyguanosine, a product of DNA oxidation and a comet assay, which is a gel electrophoresis based method for measuring DNA breaks in eukaryotic cells. Supplementation significantly decreased DNA damage markers in this study providing preliminary support for anti-oxidants in cats with CKD.²¹⁰ Overall the results of these studies only begin to touch the surface in terms of the role of oxidative stress in cats with CKD and whether any form of anti-oxidant supplementation would be of benefit. Further work is required in this area before routine anti-oxidant supplementation in cats can be advocated.

Conclusions: Given the similarities in mammalian physiology it seems likely that many of the pathophysiological mechanisms implicated in the development and progression of kidney disease from experimental and human studies are likely to be common and important in the cat. However, further studies are required that will help demonstrate the links between environmental and individual factors that predispose cats to CKD, perhaps optimizing the way that we provide health care for cats in the earlier stages of their life. An improved understanding of the factors associated with progression of disease and the key mediators of these changes may allow us to adapt our treatment strategies for cats diagnosed with CKD. Particularly in relation to proteinuria, RAAS activation and hypoxia further

clinical evidence that modulating these parameters improves outcome is required.

Figure Legends:

Figure 1: Flow diagram representing the stages involved with replicative and stress induced cellular senescence in renal ageing.

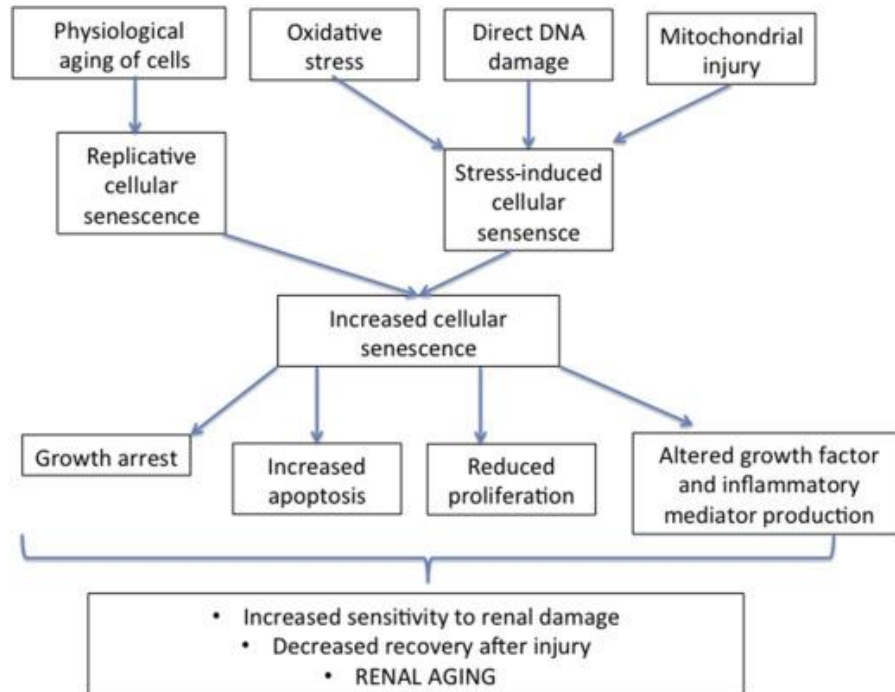


Figure 2: Diagram giving key steps in the process of renal injury, myofibroblast production and interstitial inflammation and fibrosis.

TGF β ; transforming growth factor-beta, MCP1; monocyte chemoattractant protein 1, CTGF; connective tissue growth factor, TNF; tumor necrosis factor, α SMA; alpha-smooth muscle actin, EMT; epithelial to mesenchymal transition, PDGF; platelet derived growth factor, FGF2; fibroblast growth factor 2, Ang II; angiotensin II.

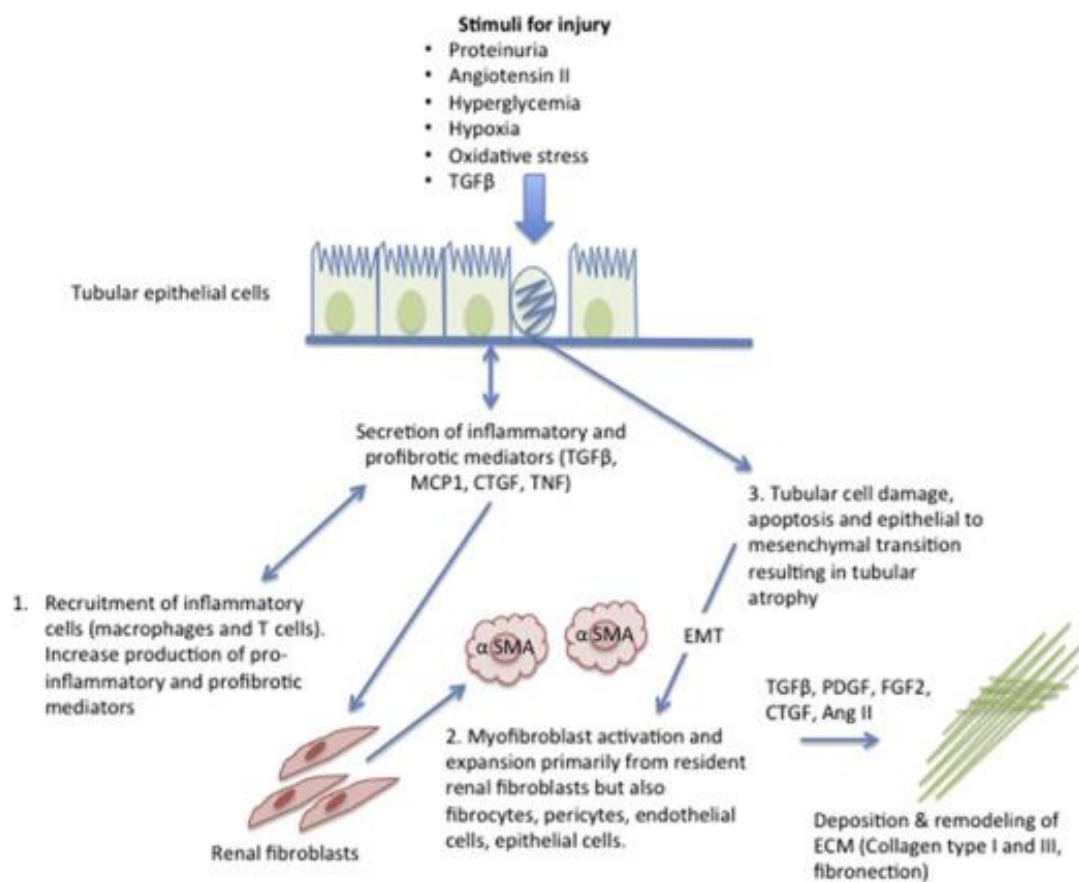
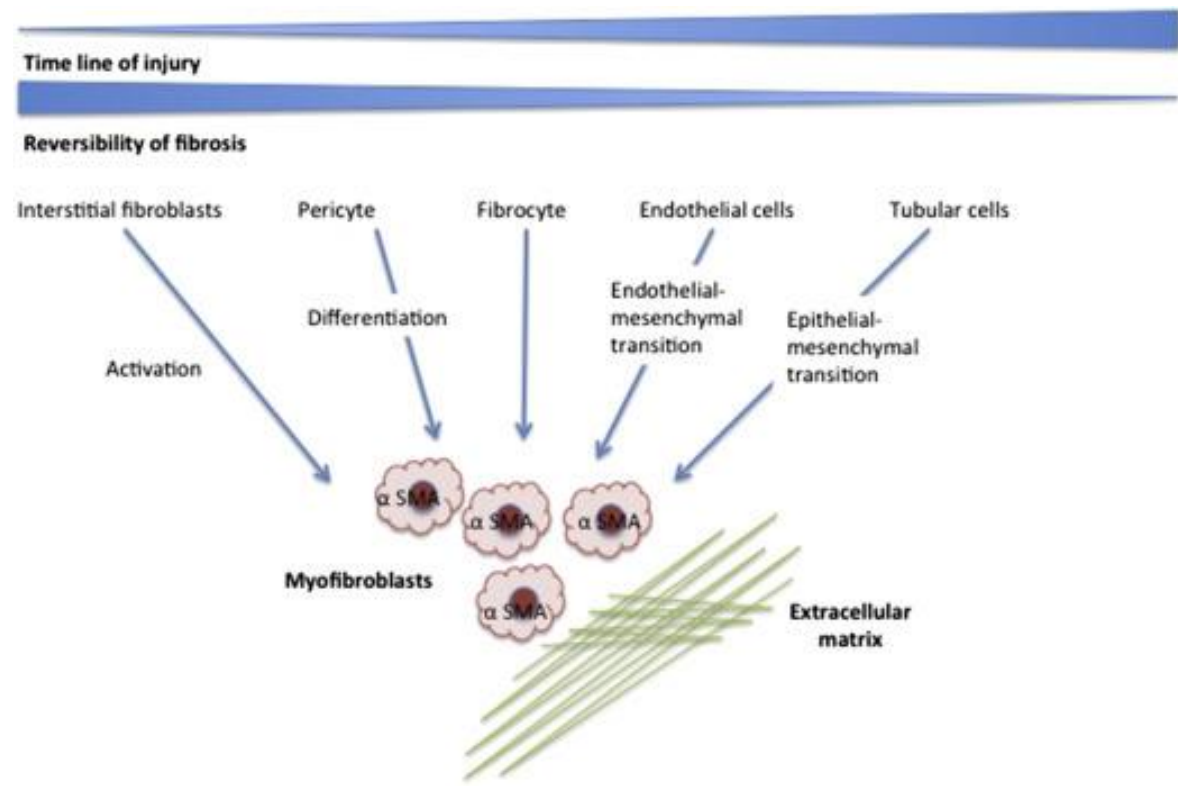


Figure 3: Diagram exploring the origin of myofibroblasts during the time-line of renal injury.



αSMA; alpha-smooth muscle actin.

Figure 4: Flow diagram of the effects of the renin-angiotensin aldosterone system on renal fibrosis and progression of kidney disease

ACE; angiotensin converting enzyme 1, ACE2; angiotensin converting enzyme 2, Ang I; angiotensin 1, Ang II; angiotensin II, Ang1-7; angiotensin 1-7, Ang 1-9; angiotensin 1-9, NFκβ; nuclear factor kappa beta, TGFβ; transforming growth factor- beta.

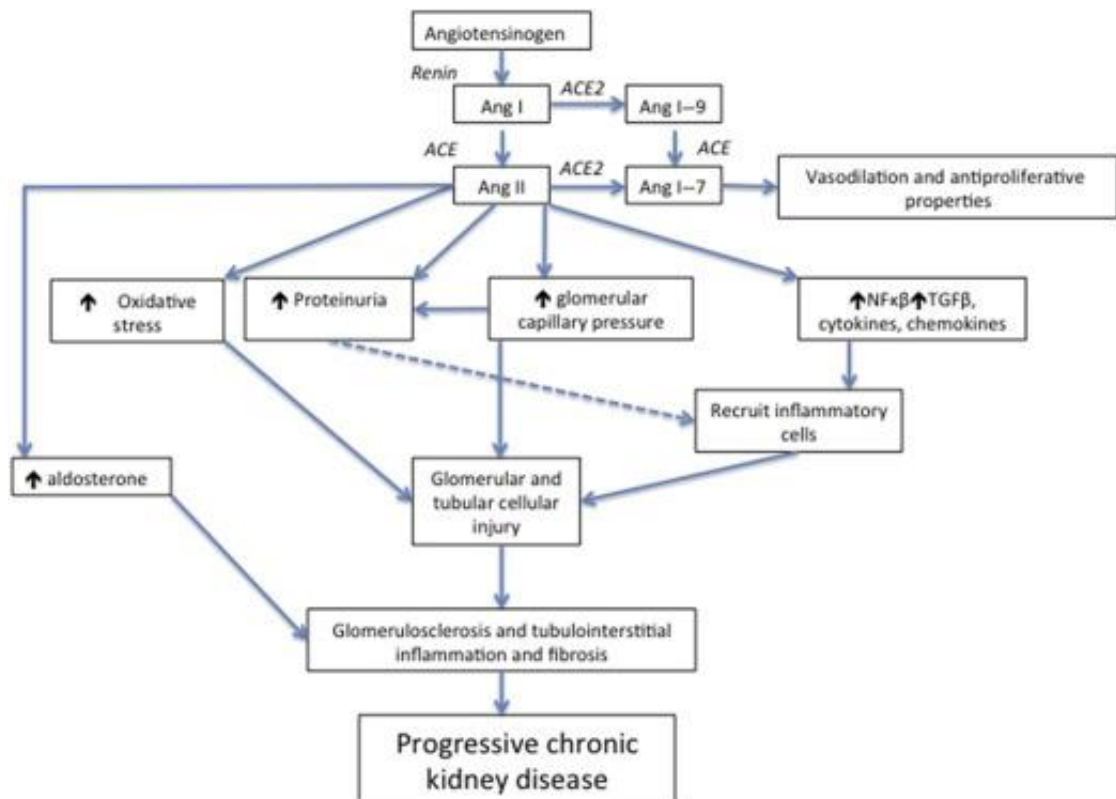
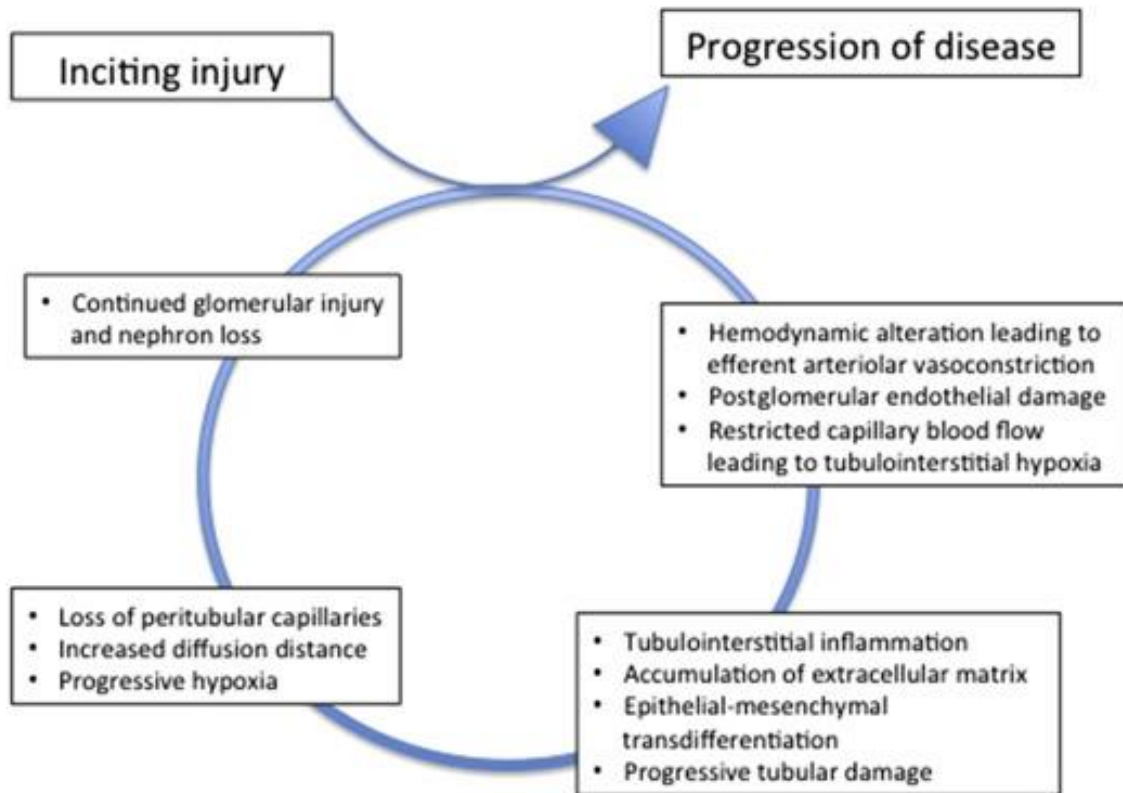


Figure 5: The role of hypoxia in progression of chronic kidney disease



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