

The Roles of Age, Glomerular Location, and Collagen Expression in the Canine Kidney:

Analysis of a Lifespan Study

By

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ABSTRACT

It is well documented that the incidence of renal disease, and therefore renal dysfunction, increases with age in many species of mammals. Such alterations in renal structure and function may significantly affect long-term toxicology studies. The purpose of this study was to assess the temporal evolution of glomerulosclerosis, an important renal lesion, in laboratory housed dogs, an important model system in chronic toxicological studies. We histopathologically examined representative sections of dog kidneys, quantified glomerular lesions (using the 0-5 scale of the World Health Organization classification system) and performed of statistical analysis of the extent and distribution of such changes. The kidney samples were obtained by necropsy, and occasionally biopsy, procedures from a collection of 159 purebred Beagle dogs maintained for their entire lifespan in well-controlled conditions. The lesions were correlated with sex, age, and intra-renal location of affected glomeruli to determine the relationship of each in the development of glomerulosclerosis. All dogs examined had some degree of glomerulosclerosis. In the youngest (up to 2 years of age), this was minimal, but was more advanced by middle age (3-7 years). The condition progressed with further aging and was associated with progressive fibrosis and tubular loss. Location and advancing age were significantly related to the development of glomerulosclerosis such that as age increases, the incidence of glomerulosclerosis increases, with the inner medullary ray and inner cortex demonstrating the highest occurrence. Using immunohistochemical analysis, the percentage of type IV collagen within glomeruli was determined. No significant increase in type IV collagen in glomeruli due to age or location was seen. An increase in type III or type V collagen within glomeruli was not apparent either, upon visual examination. This study indicates that renal lesions, including glomerulosclerosis, occur commonly and progress over the lifetime in a genetically similar population of laboratory Beagle dogs maintained under optimal standard environmental conditions. Such typical, age-related change needs to be taken into consideration when conducting chronic toxicological experiments using such animals.

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Introduction

A progressive loss of nephron mass with age occurs in virtually every mammalian species, except horses (Edwards, Legendre et al. 1987; Grauer 1992). Renal disease in dogs is common and can occur at any point in a dog's life, as can be seen both morphologically and functionally. There is evidence that diseases, including diabetes, hypertension, and hereditary nephropathy, are some causes of glomerulosclerosis, defined as a deposition of extracellular matrix (ECM) in the glomerulus of the kidney. However, a significant amount of disease contributing to the concurrent loss of renal function is non-infectious in nature, particularly in middle-age and older dogs. Robertson (2001) reported that by the age of five years, nearly 60 percent of dogs show renal lesions and decrements in renal function. In dogs ten years and older, this approached 90 percent (Robertson 2001). This loss of function in older dogs is progressive and irreversible and is typically associated with several factors, including:

- A gradual decline in glomerular filtration rate (GFR),
- An accumulation in the blood of metabolic by-products,
- Increased severity of histological lesions, and
- Development or worsening of clinical signs (Reinhart and Sunvold 1998).

Older animals commonly demonstrate generalized signs of renal disease, such as lethargy and wasting (Robertson 2001).

Despite the frequency of such cases, the question remains as to what predisposes a dog to develop renal lesions, even in the absence of systemic disease.

The hyperfiltration hypothesis, proposed by Brenner (1981) from studies in Munich-Wistar rats, describes a phenomenon that may account for renal lesions that occur with advancing age. He proposed that any disease process (in this case age) which reduces nephron mass will cause hyperfiltration and hyperperfusion of the remaining nephrons. Consequently, this will cause increased "wear and tear" and subsequent sclerosis of those nephrons and progressive fibrosis of the interstitium. The replacement fibrosis may in turn alter renal blood flow, as the presence of fibrous connective tissue begins to compromise nutrient and waste diffusion. At the same time, glomerulosclerosis leads to impaired peritubular perfusion, resulting in a loss of adequate blood flow to portions of the nephron with the highest metabolic activity (proximal and distal tubules), thereby rendering these areas more susceptible to further injury (Hostetter, Olson et al. 1981). This phenomenon may occur over the lifetime of the animal, finally resulting in end-stage renal disease (ESRD), defined as the point at which the GFR is less than 5% of normal (Michell 1988). ESRD is typically characterized by weight loss, polyuria (excessive urination), polydipsia (excessive thirst), gastrointestinal disturbances such as diarrhea and vomiting, anorexia, and nonregenerative anemia. These clinical symptoms are typical of the terminal stage of uremia (Cotran, Kumar et al. 1999).

Acute renal failure (ARF) is typically seen as a sudden reduction in GFR in all nephrons without time for adequate compensation. ARF can be linked to toxin exposure, inflammation, or severe reduction of renal perfusion (by hypovolemia or hypotension). A

full recovery without evidence of lesions is possible with ARF, although nephrons may not return to normal function (Michell 1988).

Chronic renal failure (CRF) takes longer to develop and is usually progressive, with limited compensation for lost nephrons. CRF is an irreversible process, resulting in permanent functional and morphological defects, ultimately leading to ESRD (Michell 1988; Shapiro and Schrier 1992).

Using the kidneys derived from an aging population of Beagle dogs in a controlled environment, we sought to determine the types and magnitude of spontaneous renal lesions that occur, and to quantify and analyze these lesions. The incidence of glomerulosclerosis, defined as the adherence of the glomerular basement membrane (GBM) to epithelial cells of the urinary (Bowman's) capsule, was the primary lesion of interest. We focused on glomerulosclerosis because this lesion renders nephrons inoperable, thereby significantly impairing the ability of the kidney to filter blood. The dogs in this study were genetically similar, were fed the same diet, and lived in the same area. Therefore, if renal lesions occurred in the absence of intercurrent chronic disease, a significant contributing factor to this occurrence would be the genetics of the dog itself (i.e., in every Beagle dog, there will be *age-related attrition* leading to renal failure). Several underlying causes of such morphological changes were also investigated.

Accordingly, the hypotheses were as follows:

- As age increases, the incidence of spontaneous renal lesions resulting in glomerulosclerosis increases in Beagle dogs
- The effect of the lesions may be demonstrated by decreased function, as seen in clinical tests;
- There is no effect of sex on the incidence of glomerulosclerosis in Beagle dogs;
- There is an increase in collagen deposition in spontaneous renal lesions due to aging in Beagle dogs.

There are no published data available where spontaneous renal lesions in aged Beagle dogs are correlated to age/or and sex. Mathematical analysis of renal lesions in dogs is not described in the literature. Consequently, this study may provide the first indication of whether the genetic makeup of a Beagle dog contributes to spontaneous renal lesions, such that the incidence of renal lesions can be predicted by a dog's age.

Literature Review

Clinical Signs of Renal Failure

Kidneys serve as the primary organ in the maintenance of homeostasis and waste removal. Consequently, failure of these organs results in primarily a buildup of nitrogenous wastes (*azotemia*) in the plasma, and also a failure in the regulation of extracellular fluid volume, blood pressure, and systemic pH. Hypocalcemia and anemia may also occur, as the kidneys play integral roles in the reabsorption of calcium from the diet as well as the regulation of the mass of erythrocytes (Shapiro and Schrier 1992; Cotran, Kumar et al. 1999; Robertson 1999).

Clinicians use several laboratory tests to diagnose renal failure. However, renal failure can occur without significant, immediate clinical symptoms (Solez and Finckh 1984). Three common clinical tests for assessing renal function include determination of creatinine (CRT), blood-urea nitrogen (BUN), and protein content of the urine. To diagnose renal dysfunction, several tests are usually used in concert, as no single test can give a definitive measurement of “renal failure” (Biewanga, Gruys et al. 1982).

CRT is formed as a byproduct of muscle metabolism. Nearly all CRT that enters the kidneys is excreted. Because CRT is present in the plasma at a relatively constant concentration and there is no reabsorption and minimal secretion, the excretion rate is nearly equal to the rate at which it is filtered. Therefore, under steady-state conditions, the rate of CRT excretion is equal to the rate of its production in skeletal muscles (Guyton and Hall 1998). CRT excretion generally remains constant, varying only between species on the basis of lean muscle mass. It follows then that if renal disease is present, one should see an increase in blood-plasma CRT levels.

Another commonly used measurement of kidney function is BUN, a product of the metabolism of proteins. In the blood, ammonia released from α -ketoglutaric acid (a by-product nonessential amino acid synthesis) enters the liver, where it reacts with carbon dioxide to produce urea, from the degradation, or deamination, of amino acids, and water. Consequently, nearly all urea is synthesized in the liver. After its formation, urea diffuses from the liver cells back into the blood, where it travels to the kidneys. Half the urea that enters the kidneys is reabsorbed in the proximal tubule by passive reabsorption (Guyton and Hall 1998). Urea constitutes the main nitrogenous waste component of urine (Chen 1973). Urea nitrogen values temporally vary, unlike CRT. BUN can be elevated by the consumption of a high protein diet, the administration of corticosteroids, and/or pathologic states (fever, catabolism, intestinal hemorrhage) (Gleadhill 1994).

Formation of Urine

Filtration of the blood does not occur before entering the glomerular capsule because of the non-porous quality of the endothelial cells lining the afferent arteriole. The endothelium of the glomerular capillaries, however, has numerous pores, or fenestrae, through which formed elements of the blood, such as leukocytes, platelets, and erythrocytes, cannot pass (Henrikson 1998). Blood pressure forces fluid from the capillaries through the fenestrae (Campbell 1990). The fenestrae are relatively large and therefore do not act as a major barrier to plasma proteins or the molecules bound to them (Guyton and Hall 1998).

The endothelial lining of the glomerular capillaries and mesangial matrix, provide structural support for the glomerulus. Together, they make up the first of three layers that compose the filtration barrier of the glomerular capsule. The GBM separates the innermost endothelial and mesangial cells from the outermost layer of the barrier, the podocytes. Podocytes are so named because each cell body has elongated processes that wrap around the capillary (Henrikson 1998).

Large amounts of water and small solutes pass effectively through the GBM, but passage of plasma proteins is prevented, due largely to the membrane's composition. The lamina rara interna and the lamina rara externa make up the inner and outer layers of the GBM, respectively. The lamina rara interna acts as a filter for macromolecules of a particular size, i.e. those that are too large do not filter. The lamina rara externa mainly aids the function of the middle layer, or lamina densa, of the GBM. These layers are principally composed of type IV collagen, laminin, and fibronectin and have a slight negative charge (Henrikson 1998).

The electron dense lamina densa allows filtration of macromolecules of a particular charge. The negative charge repels anionic molecules in the plasma, in effect preventing them from crossing the barrier. The type IV collagen concentration in the lamina densa, as well as proteoglycans that compose the lamina rarae interna and externa creates this charge. Research has shown that mesangial cells phagocytize some molecules filtered here by the GBM. In addition to providing structural support and engulfing this filtrate, mesangial cells are also believed to aid in the control and passage of blood through the glomerular capillaries by the production of vasoactive agents (Henrikson 1998).

The outermost layer of the filtration barrier of the glomerular capsule is composed of visceral epithelial cells called podocytes. The "foot processes," or pedicles, lie adjacent to one another around the capillary. Narrow spaces, termed slit pores or filtration slits, between pedicles allows for further filtration. Recent evidence suggests that some molecules filtered here are phagocytized by the podocytes (Selkurt 1976; Henrikson 1998).

Normally, very little protein is present in the urine as a result of anionic proteins being repelled by the negatively charged lamina densa (Barsanti and Finco 1979). The passage of neutral macromolecules is inhibited by the size barrier of smaller slit pores on the outermost layer of the GBM (Guyton and Hall 1998). As proteins decrease in size,

they are filtered in increasing amounts. This size barrier approaches that of albumin, the smallest of proteins (Bergstein 1999). The majority of proteins in the filtrate is reabsorbed in the proximal convoluted tubule (PCT) and are hydrolyzed by lysosomal enzymes (Gasse and Verniory 1977). Charge depletion may contribute to proteinuria by altering the size-selective properties of the GBM, which probably plays a secondary role (Avram 1985).

Proteins that appear in the urine then, have not been reabsorbed by the PCT. In a pathological state, the presence of proteins may indicate lesions in either the tubules, the glomerulus, or both—in which case large and small proteins are excreted (Lillehoj and Poulik 1986). Tubular proteinuria is characterized by detection of large amounts of low-molecular weight (LMW) proteins, such as Ig light chains, albumin, and β_2 -microglobulin, in the urine as a result of damage to the PCT such that it does not function at full reabsorptive capacity. The more common glomerular proteinuria is a result of increased permeability of the GBM. It can be divided into two subsets: selective (loss of proteins up to and including albumin) and nonselective (loss of larger molecular weight proteins). Glomerular proteinuria is typically an early sign of kidney disease; at the same time, it plays a role in the progression of damage to the kidney (Bergstein 1999).

Numerous studies have found a direct correlation between the degree of proteinuria, an abnormality in kidney function that results in urinary protein excretion exceeding 2-3g/day, and the rate of progression of renal failure (Cameron 1990). Proteinuria may be transient or persistent, resulting from nonpathological (fever, exercise) or pathological (tubular or glomerular) processes, respectively (Bergstein 1999). The mechanism of febrile-induced proteinuria is unknown (Waller, Ward et al. 1989). During exercise, GBM permeability is thought to increase due to vasoconstriction of renal arterioles causing a decrease in GFR, such that the diffusion of macromolecules into the tubular lumen is enhanced (Poortmans 1985). Persistent proteinuria usually results from disorders that increase GBM permeability, such as systemic lupus erythematosus, or genetic defects, such as Alport syndrome (Bergstein 1999). The more common cause of proteinuria is a result of increased permeability of the GBM. The abnormal passage of proteins across the glomerular capillary wall brings them into contact with tubular cells. As the tubular cells reabsorb the proteins, they may be exposed to agents (e.g., complement components, transferrin, lipoproteins) that are directly toxic to the cells. In addition, a number of genes may become activated, leading to the production of growth factors, vasoactive and inflammatory agents, such as endothelin-1, leading to tubulointerstitial lesions. Therefore, while glomerular proteinuria is typically an early sign of kidney disease, it may play a role in the progression of damage to the kidney at the same time (Bergstein 1999).

Overview of Glomerulosclerosis

Age-related changes in renal structure and function are well documented in both man and rats. In man, the percentage of sclerosed glomeruli increases from 0.1% at age 1 year to over 10% by the seventh decade of life. In rats, global glomerulosclerosis (encompassing the entire renal cortex) as well as a focal and segmental sclerosing processes involving other glomeruli takes place. Rats commonly have sclerotic lesions as

they age, and also have significant proteinuria when these lesions are present (Kaysen and Myers 1985). Couser and Stilmant (1976) observed expansion of the mesangial matrix, thickening of the basement membrane, epithelial cell foot process fusion, adhesions to the urinary capsule, and eventually capillary wall wrinkling and collapse in humans with glomerulosclerosis (Couser and Stilmant 1976).

All of the complex details involved in the pathogenesis of glomerulosclerosis are not known. One current hypothesis highlights the effect of glomerular cell changes on podocytes, which is then followed by capillary loop collapse along with adhesions between the capillary tuft and Bowman's capsule (Bariety, Nochy et al. 1998). A second hypothesis proposes that responses manifested by glomeruli in glomerulosclerosis are analogous to wound healing (Harris 1999). While the pathogenetic mechanisms appear to be multiple, the final outcome is widely held as resulting in an increased amount of mesangial extracellular matrix, leading to progressive distortion of glomerular architecture (Striker, Doi et al. 1989).

Podocytes are normally involved in processes such as GBM turnover, maintenance of the filtration barrier, and support of the glomerular tuft. Further, podocytes also act as a support structure to the GBM-mesangial cell system. Notably, mesangial cells and podocytes are mutually dependent on each other for support; if one of them fails, the other will necessarily be affected. Podocyte foot processes, composed of microfilament bundles containing α -actin, α -actinin, and myosin, are firmly attached to the GBM by a chain of vinculin and talin proteins linking the actin to the underlying GBM via cell membrane-associated integrins. Each of these processes is capable of locally counteracting the distension of the area of GBM underlying it. This mechanism may be crucial in cases of glomerular injury that may result in an increase in glomerular capillary pressure. Since podocytes are terminally differentiated cells, they are unable to undergo cytokineses after ontogenesis, limiting the podocyte's capacity to cope with increased workload. Consequently, upon exposure to increased glomerular capillary pressure, podocytes undergo changes in a fairly stereotypical manner. These changes initially involve foot process effacement, cellular hypertrophy (microscopically represented by multinucleated cells), cell body attenuation, cytoplasmic overload with a reabsorption of droplets, and finally, detachment from the GBM (Kriz 1998).

Glomerulosclerotic lesions become irreversible once they have progressed beyond these initial podocytic changes (Bariety, Nochy et al. 1998). Podocyte detachment results in ballooning of the peripheral capillaries and contact of the denuded GBM with the parietal epithelium of the urinary capsule, eliciting a reaction in which the epithelial cells attach to the GBM. As these adhesions progressively enlarge, physical forces upon the involved capillary loop will markedly change, initiating further podocyte damage. The result is the development of a sclerotic lesion. Consequently, glomerular capillaries either become trapped or occluded and undergo segmental tuft collapse (Floege and Grone 1998; Harris 1999).

A second morphologically and biosynthetic distinct zone of extracellular matrix within the glomerulus lies within the mesangial matrix, normally composed of collagens type IV, V, and VI (Wardle 1992). Contact of mesangial cells with this matrix ensures that the cells do not proliferate. The state of contractility of the mesangial cells regulates

the area of the GBM available for filtration (Striker, Doi et al. 1989; Schreiner 1990; Wardle 1992). Normally, transforming growth factor- β (TGF- β) is in a bound form attached to heparan sulphate proteoglycans and type IV collagen. Several factors may trigger the leakage of macromolecules and fibronectin into mesangial areas, thereby causing mesangial cell proliferation. These include physical stress on capillary walls, alterations in mesangial architecture due to the deposits of abnormal proteins (as may occur in diabetes or immune-complex deposition), the presence of abnormal types or levels of lipoproteins, and, finally, the periglomerular or intraglomerular accumulation of leukocytes, particularly macrophages. Hyperlipidemia may exacerbate mesangial cell proliferation as low-density lipoproteins (LDLs) can directly bind anionic glycosaminoglycans of the GBM or mesangial matrix, thereby neutralizing the filtration barrier. Furthermore, LDLs have been shown to induce mesangial cell proliferation and may be cytotoxic, particularly when oxidized (Schreiner 1990; Wardle 1992). These changes in glycosaminoglycans can result in the liberation of TGF- β by mesangial cells, favoring formation of type I collagen as well as mesangial cell proliferation (Wardle 1992).

A likely early event initiating the inflammatory process in glomerular injury is triggered by insult to the glomerular endothelial lining (El Nahas 1998). The activation of monocytes and their subsequent release of cytokines such as interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α) can transform the endothelial lining from an anticoagulant surface to a procoagulant one. This may lead to microthrombi formation, hyaline deposition, increased permeability of the glomerular endothelium, and possibly proliferation of mesangial cells. Platelets may be activated and release platelet-derived growth factor (PDGF), which stimulates mesangial cell proliferation (Schreiner 1990). Further, autocrine loops may be initiated if mesangial cells are stimulated to release PDGF, while concurrently upregulating their PDGF receptors, resulting in further proliferation. These dedifferentiated mesangial cells synthesize types I and III collagens (El Nahas 1993; Harris 1999). Phenotypic changes of mesangial cells have been postulated to explain the expression of α -smooth muscle actin (α -SMA) within scarred glomeruli, possibly explaining the appearance of interstitial collagen within these glomeruli (El Nahas 1995).

Collagen Structure

Collagens are a family of proteins that make up the major structural components of the extracellular matrix (ECM). At least 18 different types of collagen have been identified. The existence of so many types of collagen allows the formation of various structures--fibers, filaments, sheet-like structures, and/or anchoring fibrils--depending on the tissue in which the collagen is located. The monomeric building block of three polypeptide α -chains, consisting of repeated *Gly-Xaa-Yaa* sequences interacting to form a triple helix, is a common theme found in all collagens (Piez 1976).

In almost all cases, *Xaa* and *Yaa* represent proline and hydroxyproline, respectively. The presence of glycine is essential in the proteins, as it is the only amino acid with a side chain small enough to fit into the center of the helix; proline and

hydroxyproline residues exist outside the triple helix (Tryggvason 1995). Hydrogen bonds link the amino peptide bond of glycine with a carbonyl peptide group in an adjacent polypeptide, helping to hold the chains together (Lodish, Berk et al. 1999). Collagen is initially synthesized intracellularly on ribosomes in a precursor form as procollagen (Piez 1976). Assembly of the procollagen chains into the triple helix follows hydroxylation and glycosylation of certain residues, after which procollagen is secreted into the extracellular space. Shortly after secretion from the cell, the extensions of procollagen, termed propeptides, are cleaved by collagen peptidases, promoting the formation of fibrils, called tropocollagen (Cotran, Kumar et al. 1999). Proteases target the propeptides because the N- and C-termini have low hydroxyproline content and little or no glycine and proline (Piez 1976). Oxidation of specific lysine and hydroxylysine residues usually accompanies tropocollagen formation, resulting in cross-linkages between α -chains of adjacent molecules. This cross-linking is a major contributor to the tensile strength of collagen (Cotran, Kumar et al. 1999).

Types of Collagen

The most common collagen is type I, which forms fibrils of high tensile strength. Type I collagen is typically found in skin, tendons, ligaments and other types of connective tissue that require high tensile strength for their function. Structurally, type I collagen is composed of two $\alpha 1$ chains and one $\alpha 2$ chain packed together side-by-side. These chains are assembled into fibrils through their head-to-tail arrangement and laterally staggered alignment (Tryggvason 1995). Fibrils are finally packed in parallel bundles, forming fibers. Cross-links between two residues at the C-terminus of one collagen molecule with two similar residues at the N-terminus of an adjacent molecule stabilize the side-by-side packing (Lodish, Berk et al. 1999).

Collagen types II and III are each composed of three identical α -chains and also form a fibrillar structure similar to that of type I. Whereas type II collagen is usually found in cartilage, type III collagen is found in blood vessels and also colocalizes with type I as a minor constituent of skin. Collagens types II and III form fibers that are present in various tissues where they provide moderate tensile strength (Piez 1976; Tryggvason 1995).

Structurally speaking, one of the most complex collagens is type IV collagen. This collagen is composed of six α -chains that self-assemble after secretion into the extracellular space to form a sheet network (Tryggvason 1995). Each α -chain is composed of an approximately 15-residue noncollagenous amino terminus (7S domain), approximately 1400 residues of Gly-Xaa-Yaa repeats which are interrupted in several sites by noncollagenous sequences, and an approximately 230-residue noncollagenous segment (NC1 domain) at the carboxyl terminus (Mariyama, Leinonen et al. 1994). Several cross-linking sites in the 7S domain allow lateral associations to form between chains, which align alternatively in a parallel/anti-parallel fashion, forming tetramers. In addition, the arrangement of anti-parallel molecules in this domain allows maximal hydrophobic interactions between opposing strands. The 7S domain of each α -chain contains varying numbers of cysteine residues that participate in intra- and intermolecular

disulfide bonds, promoting stabilization of the arrangement. Intramolecular bonds within each α -chain form in the more central region of the chain, near a cleavage site for a type IV specific collagenase (Timple 1989).

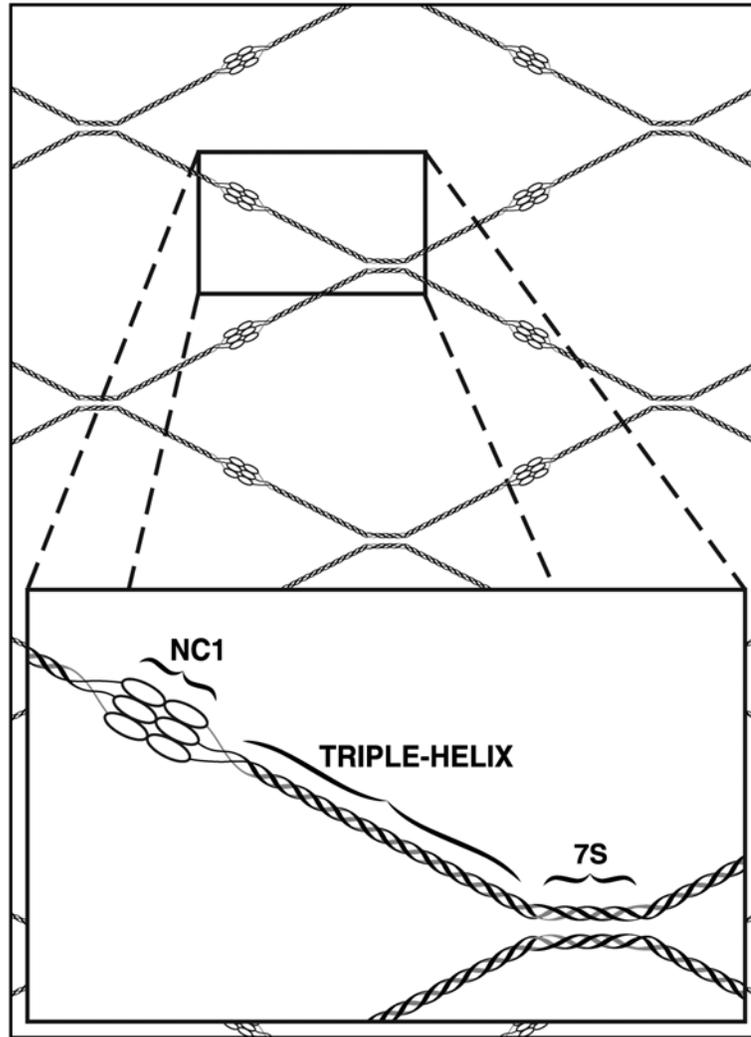


Figure 2. The interaction of the NC1 domain and cross-linking 7S domains of two separate type IV collagen molecules contributes to the stabilization of the sheet-like network.

Interchain disulfide bonds at cysteine residues stabilize the dimerization of two monomers of type IV collagen, contributing to further stabilization of the entire network (Timple 1989; Hudson, Reeders et al. 1993; Olsen and Ninomiya 1999). Evidence indicates that association of like kinds of monomers is favored (Hudson, Reeders et al. 1993). Further studies suggest that the formation of collagen IV dimers is accompanied by conformational changes within the NC1 domain, as only dimers have the ability to reform intact globular structures (Timple 1989). Thus, the specificity for assembly of triple helical monomers appears to be provided by the NC1 domain of each α -chain. In addition to these interactions, the triple helical domains intertwine and interact with NC1

domains to form the supercoiled structure. The flexibility required for supercoiling is hypothetically provided by the short noncollagenous interruptions, unique to type IV collagen, in the triple helix (Hostikka, Eddy et al. 1990; Hudson, Reeders et al. 1993). These interruptions vary in length, pattern, and number between the six type IV collagen chains (Mariyama, Leinonen et al. 1994).

Some limitations are imposed on how the α -chains of type IV collagen can self-assemble. Immunohistochemical and RNase protection assays demonstrate that $\alpha 3$ and $\alpha 4$ chains are consistently coexpressed and appear only in basal laminae (BL) that are $\alpha 5(\text{IV})$ positive. These findings are consistent with studies showing that the NC1 domains of $\alpha 3$ and $\alpha 4$ chains associate with each other and that their expression is coregulated (Miner and Sanes 1994). Quantitative immuno-electron microscopic studies further show that $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains can form triple helices together, but not with $\alpha 1$ or $\alpha 2$ chains. Furthermore, the absence of $\alpha 5$ makes incorporation of $\alpha 3$ or $\alpha 4$ chains into basement membranes impossible (Furness 1997).

Type V collagen is a heterotrimeric molecule composed of three different α -chains. As type V collagen has a fibrillar structure, it can be found in bone, skin, and tendon. Unique to this type of collagen is the ability to form a mixed heterotrimer with type XI collagen, as both types have similar structures (1999).

Type IV Collagen Expression

Type IV collagen is one of the major components of all basement membranes. Basement membrane is found under epithelial and endothelial cell linings, surrounding cells (specifically nerve, muscle, and fat) and as a filtration barrier for macromolecules in the placenta, blood-brain barrier, and kidney (Glanville 1987). Within the mature kidney, type IV collagen is normally found in the GBM, mesangial matrix, tubular basement membranes (TBM) and basal laminae (BL) of blood vessels. Each α -chain of type IV collagen exhibits regional specificity within the kidney (Olsen and Ninomiya 1999). More specifically, in adult mice, Miner and Sanes (1994) found anti-collagen $\alpha 1$ and $\alpha 2(\text{IV})$ antibodies stained all TBM, the glomerular mesangial matrix, and BL of blood vessels intensely, but stained GBM poorly. On the other hand, $\alpha 3$ - $\alpha 5(\text{IV})$ antibodies stained GBM intensely and only cortical and medullary TBM; there was no staining of the mesangium or blood vessel BL. No significant difference in localization of $\alpha 3$ - $\alpha 5(\text{IV})$ chains was detected, suggesting that these chains co-localize (Miner and Sanes 1994).

Furthermore, the α -chain composition of type IV collagen varies during glomerular development. Miner and Sanes (1994) found a progression of collagen IV chain expression in the GBM of newborn rats. Immunohistochemical staining for type IV collagen showed that in newborn rats, GBM in all but the most mature glomeruli contained $\alpha 1$ and $\alpha 2(\text{IV})$ chains, while the BL capillary loops contained $\alpha 3$ - $\alpha 5(\text{IV})$. However, in the most mature glomeruli, collagen $\alpha 1$ and $\alpha 2(\text{IV})$ were no longer abundant in the GBM, but were present at high levels in the mesangial matrix. These

results suggest a progression of type IV collagen production such that only $\alpha 1$ and $\alpha 2(\text{IV})$ become concentrated in the mesangium while $\alpha 3$ - $\alpha 5(\text{IV})$ accumulate in the GBM, replacing the $\alpha 1$ and $\alpha 2$ chains initially present (Miner and Sanes 1994).

Using the Samoyed dog model to study Alport syndrome, Harvey et al. (1998) found the presence of a “developmental switch” in type IV collagen expression changing a fetal GBM to an adult GBM. Whether this switch acts at the protein, mRNA, and/or the gene levels is not known. Specifically, Alport syndrome is characterized by a nonsense mutation in the COL4A5 gene (*COL4A5*) encoding $\alpha 5(\text{IV})$ such that the $\alpha 5$ chain cannot be synthesized correctly. In affected dogs, positive staining for only $\alpha 1$ and $\alpha 2(\text{IV})$ chains was found in the mesangial matrix, GBM, and the urinary capsule. In normal canine glomeruli, positive staining for $\alpha 1$ and $\alpha 2(\text{IV})$ chains occurred only in the mesangial matrix while the GBM contained primarily $\alpha 3$ - $\alpha 5(\text{IV})$ chains. The investigators found positive staining for $\alpha 3$ - $\alpha 5(\text{IV})$ chains upon initial appearance of capillary loops in glomeruli. Prior to this stage, $\alpha 1$ and $\alpha 2$ chains predominated, confirming active remodeling of the GBM during development. Significantly, neither $\alpha 3$ nor $\alpha 4(\text{IV})$ chains were seen in the GBM of affected dogs since mutations of *COL4A5* disrupted assembly of the $\alpha 3$ and $\alpha 4$ chains. Further support for the idea of control at the gene expression level comes from the finding that this mutation resulted in >77% reduction of mRNA levels for not only the $\alpha 5$ chain, but the $\alpha 3$ and $\alpha 4$ chains as well (Harvey, Zheng et al. 1998).

Collectively, the data imply that the $\alpha 1/\alpha 2(\text{IV})$ network is essential for normal glomerular development and the $\alpha 3/\alpha 4/\alpha 5(\text{IV})$ network is essential for long-term maintenance of glomerular structure and function. Persistence of the fetal network predisposes the GBM to proteolytic degradation, resulting in deterioration of kidney function over time as the GBM splits. This condition ultimately results in end-stage renal disease, as filtration process cannot occur properly (Harvey, Zheng et al. 1998).

Collagen Metabolism

Collagen metabolism is affected in a variety of pathological conditions affecting different tissues. Mutations of DNA may have an effect on different domains of the collagen molecule, thereby affecting function. Some mutations may be tissue-specific, and affect on the structural function of the protein in the tissues. Consequently, various kidney diseases result from different factors affecting collagen expression and metabolism. In glomerulosclerosis for example, expression and accumulation of collagen types normally not present, such as type I collagen (which is normally found in connective tissue rather than cartilage or basement membranes), takes place (Tryggvason 1995). Diffuse glomerulosclerosis is identified by increased type IV collagen deposition in the mesangial matrix, which is typically present in only small amounts there (Couser and Stilmant 1976).

Essentially all forms of glomerulosclerosis leading to end-stage renal disease (such as diabetic nephropathy and membranous glomerulonephritis (MGN)), are characterized by an increased amount of type IV collagen (Striker, Esposito et al. 1997).

For example, in diabetic nephropathy (which is characterized by mesangial sclerosis), an accumulation of $\alpha 3/\alpha 4/\alpha 5(\text{IV})$ chains occurs, while $\alpha 1/\alpha 2(\text{IV})$ chains are present in the expanded mesangium and are decreased in the GBM. Striker et al. (1997) showed that mesangial sclerosis in diabetes mellitus and renal cancer is associated with an increase in glomerular $\alpha 2:\alpha 3(\text{IV})$ collagen mRNA ratio. However, in membranous glomerulonephritis (MGN), which is characterized by major sclerotic changes in the peripheral GBM, distribution of both $\alpha 2$ and $\alpha 3(\text{IV})$ collagen is preserved in the peripheral basement membrane. Striker et al.'s results suggest that different molecular mechanisms and/or cell types, such as glomerular and mesangial cells, are involved in the accumulation of ECM in the glomerular vs. peripheral basement membrane (Striker, Esposito et al. 1997). Autoimmune and fibrotic diseases may also result in the deposition of basement membrane components in the interstitium from which they are normally absent, as a result of the release of pro-fibrotic cytokines, such as TGF- β , from infiltrating inflammatory cells (Furness 1997).

The Role of TGF- β in Glomerulosclerosis

TGF- β is a primary mediator of tissue repair following injury. This substance has diverse range of actions on mammalian cells, including increasing matrix production while inhibiting matrix degradation, resulting in accumulation of collagen (Border and Noble 1993). This, along with TGF- β 's anti-inflammatory effects of the cytokine, is an extremely beneficial process in the case of wound repair (the "bright side" of TGF- β), but may become deleterious when it occurs over an extended period of time, especially in the glomerulus (the "dark side" of TGF- β). Consequently, failure to *terminate* the repair process in the glomerulus may set the stage for progressive glomerulosclerosis (Border, Noble et al. 1992).

Kitamura and Suto (1997) explored the role of TGF- β in tissue repair within the kidney. The antiproliferative actions of this cytokine on glomerular cells may prevent a buildup of glomerular cells, which would contribute to collapse of the capillary tuft. This could occur through modulation of the cell-cycle machinery, or by inhibition of cytokine release from activated macrophages. Similarly, TGF- β may also serve as an autocrine stimulator of mesangial cell apoptosis, preventing accumulation of the mesangial matrix (Kitamura and Suto 1997). Evidence implies that the mesangial matrix controls the behavior of mesangial cells and maintains their differentiated phenotype in the glomerulus, suggesting TGF- β is an important regulator in ECM homeostasis (Kitamura, Shirasawa et al. 1994).

TGF- β is known to counteract the actions of IL-1 and TNF- α . Both of these cytokines induce mesangial cell proliferation and an influx of leukocytes, leading to destruction of glomerular structure. TGF- β also acts as a macrophage deactivator, suggesting it acts as a "defender" against macrophage-mediated glomerular injury (Kitamura and Suto 1997).

Border et al. (1992) have provided an overview of the similarity of action of TGF- β in cases of wound repair and glomerulonephritis. Following mechanical or

immunological insult, TGF- β and PDGF are immediately released, which stimulates mesangial cell proliferation by degranulating platelets. Activated monocytes and macrophages, as well as glomerular cells, may serve to release additional TGF- β , resulting in an increase in fibronectin and proteoglycan production in the glomerulus, just as in wounded skin. However, in the glomerulus, healing of the mesangial matrix to its normal state is followed by the buildup of an excess of ECM. Regardless of the site of insult, the influence of TGF- β on other cytokines and growth factors is thought to determine the degree of mesangial cell proliferation (Border, Noble et al. 1992).

Border et al. (1990) demonstrated that antibodies to TGF- β 1 markedly reduce the degree of glomerular damage and matrix accumulation in an experimental model of acute membranous proliferative glomerulonephritis (Border, Okuda et al. 1990; Kitamura and Suto 1997). TGF- β 1 was found to induce the expression of collagen IV genes and collagen IV mRNA in NIH-3T3 cells (a fibroblast-like line used to study fibrillar collagen regulation) and in normal rat kidney cells. Therefore, not only is collagen IV mRNA accumulation mediated, at least in part, by increasing transcription of the collagen IV gene, but TGF- β 1 plays an integral role in this process (Grande, Melder et al. 1993). Grande et al. (1997) went further to determine that TGF- β 1 and other cytokines and growth factors known to be elaborated following tissue injury have no synergistic or additive effects, indicating that TGF- β 1 is the predominant mediator of collagen type IV overproduction (Grande, Melder et al. 1997).

The mechanism by which TGF- β 1 induces synthesis of type IV collagen is not clear. Grande et al. (1993) have proposed a theory that TGF- β 1 induces type IV collagen mRNA through the synthesis of another unidentified protein. In particular, the effect of TGF- β 1 on collagen type I gene expression has been linked to an NF1 site on the promoter. This same interaction may reasonably be presumed to take place when TGF- β acts on the collagen type IV gene. However, the bi-directional promoter shared by two α -chains of type IV collagen lack an NF1 site, containing only an SP1 site and CCAAT motifs. Therefore, TGF- β 1 must interact with a regulatory element to induce collagen IV gene expression (Grande, Melder et al. 1993).

Secondary Cytokines in Glomerulosclerosis

One possible step in the induction of proinflammatory cytokines is the release of endothelin-1 (ET-1), a powerful vasoconstrictor, as a result of protein stress. Glomerular injury from either antigen-antibody reactions, deposition of immunoglobulins, toxins, or cell-mediated reactions, for example, exposes resident cells (primarily proximal tubules) to urinary proteins. This exposure results in synthesis of vasoactive and proinflammatory mediators, such as ET-1. When secreted in excessive amounts, these mediators promote recruitment of monocytes and macrophages. Binding of ET-1 to receptors on monocytes/macrophages may also further induce the production of proinflammatory cytokines. Bruzzi et al. (1997) found tubular overexpression of ET-1 well before glomerulosclerosis was apparent, indicating that ET-1 precedes the release of TGF- β 1. Autoinduction of ET-1 has been described in human endothelial and PCT cells and in rat mesangial cells, resulting in a primary amplification loop (Bruzzi, Corna et al. 1997).

Further amplification may occur with the continuous release of TGF- β , leading to a cycle of persistent production and deposition of ECM (Border and Noble 1993).

Resident glomerular cells are also possible contributors to the up-regulation of collagens types I and III mRNA, or interstitial collagens. Mesangial cells produce interstitial collagens *in vitro*, but not *in vivo*. At least three mechanisms may be proposed by which intrinsic glomerular cells undergo a phenotypic change to up-regulate interstitial proteins, one of which being an increased “translatability” of interstitial collagen mRNA. Factors specific for glomerular cells, including TGF- β 1, may influence this up-regulation. Macrophages also participate in the initiation and development of glomerular injury in focal glomerulosclerosis (FGS) by the secretion of soluble regulators, such as TGF- β or IL-1, which stimulate ECM production by resident glomerular cells (Ebihara, Suzuki et al. 1992).

Another integral role played by TGF- β in collagen metabolism is in the inhibition of matrix degradation (Border and Noble 1993). In normal degradation and ECM turnover, the plasminogen/plasmin system, which is regulated by the balance between plasminogen activators (PA) and plasminogen activator inhibitors (PAI), plays a key role. Plasmin is activated by PA, inducing fibrinolysis; this activity is regulated by the production of PAI from endothelial cells, which block fibrinolysis by inhibiting the binding of PA to fibrin (Cotran, Kumar et al. 1999). TGF- β has been shown to decrease the synthesis of PA while stimulating the production of PAI, contributing to fibrin accumulation in diseased kidneys (Border and Noble 1993). Diminished synthesis of PA and/or increased production of PAI may result in an imbalance in the extracellular proteolytic process, leading to the progression of glomerular sclerotic lesions (Yamamoto, Loskutoff et al. 1998).

Recent studies indicate that angiotensin II (Ang II), a vasoconstrictive hormone central to the regulation of blood pressure, may also contribute to the renal scarring in chronic kidney disease through the induction of TGF- β 1 (Wolf 1998). Circulating angiotensin-converting enzyme (ACE) converts angiotensin I to Ang II, thereby acting as a “source” of Ang II (Cotran, Kumar et al. 1999). Myofibroblasts, which play a major role in fibrous tissue formation and wound contraction during tissue repair, express Ang II receptors. Sun et al. (1999) demonstrated that administration of an ACE inhibitor or AT₁ receptor antagonist significantly reduces TGF- β 1 synthesis, resulting in marked attenuation of renal fibrosis and preservation of kidney function. These studies support the theory that Ang II promotes fibrinogenesis through the stimulation of TGF- β 1 synthesis and release via AT₁ receptors (Sun, Zhang et al. 1999). Other studies show that Ang II contributes directly to tubulointerstitial fibrosis by the direct stimulation of ECM protein synthesis. Thus, evidence points to Ang II acting as a profibrogenic cytokine, inducing the proliferation of tubular cells, glomerular mesangial and endothelial cells, interstitial fibroblasts, and transcription of collagen IV likely through the release and autocrine actions of cytokines such as IL-6, ET-1, and PDGF (Wolf 1998). Yamamoto et al. (1998) found dramatically elevated concentrations of active plasma PAI-1 and PAI-1 mRNA in lupus-prone mice as a function of age. This increase in PAI-1 activity correlates with fibrin deposition in renal microvasculature and with the progression of systemic lupus erythematosus (SLE), a disease characterized by ECM

accumulation and the formation of glomerular, tubular, vascular, and interstitial lesions. Such lesions are histologically similar to those seen in glomerulosclerosis, suggesting an increase in PAI-1 synthesis as a contributing factor in glomerulosclerosis as a function of age. Cells contributing to PAI-1 synthesis include endothelial cells, mesangial cells, tubular epithelial cells, and infiltrating mononuclear cells (Yamamoto, Loskutoff et al. 1998).

Summary of Literature

The development of glomerulosclerosis appears to occur when the glomerulus is injured in one of several ways, resulting in a release of cytokines, such as TGF- β , IL-1 and TNF- α . In turn, the release of these cytokines causes a cytokine cascade, including the release of PAI-1, PDGF, and/or proinflammatory ET-1. In total, the actions of these cytokines leads to excessive formation of the ECM, as there is an increase in ECM formation while at the same time, matrix degradation is inhibited.

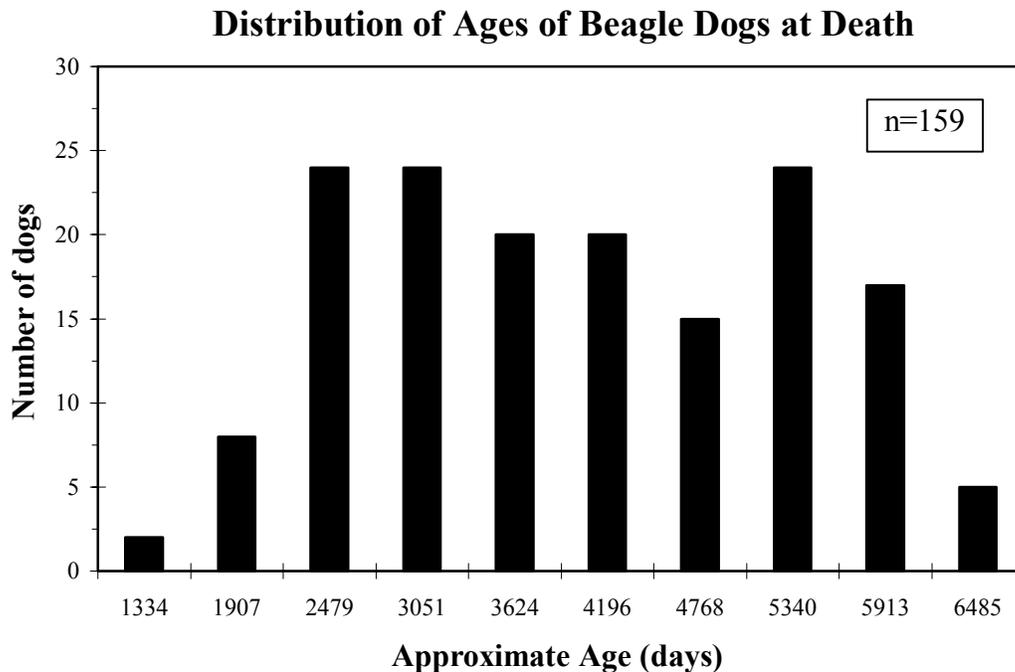
The primary type of collagen deposited in the glomerulus due to spontaneous renal lesions is unknown. It is suspected to be type IV, as it is the main component of basement membranes. Studies have shown that type IV is indeed overly deposited in cases of systemic disease, such as diabetes nephropathy.

Materials and Methods

Study animals

Kidney samples were obtained at necropsy by biopsy from 159 genetically similar male and female Beagle dogs. The ages of the dogs ranged from 762 days to 6485 days.

The study population consisted of male and female purpose-bred laboratory Beagle dogs. The age distribution of the dogs (84 males, 75 females) analyzed for renal lesions has a normal distribution with a mean and median age of 3731.7 days (± 1319.2 days) and 3679.5 days for males, and 3717 days (± 1315.2 days) and 3676 days for females, respectively (Graph 1).



Graph 1. The ages of death of all 159 Beagle dogs shows a normal distribution. The dogs either died of natural causes or were euthanized for humane reasons.

The dogs were maintained for their entire lifespan in controlled conditions at Colorado State University (CSU). They were raised as a control group in a study of radiation effects on Beagle physiology. The dogs were fed a 16.9% protein diet and lived outside with cement doghouses for shelter, in proximity to the other dogs, but segregated from wild animals to prevent the spread of disease. They were annually examined and vaccinated for rabies and DHLPP. Any illness noticed by the staff was treated accordingly. Most dogs died of natural causes; others were euthanized for humane reasons, such as cancer.

Slide Preparation and Grading

Two hundred sixty-nine (269) hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stained slides were prepared from these kidney samples. Lesions in these sections were detected and recorded. First, a blinded reading was conducted, such that the assessment of each slide was made without knowledge of the age or sex of the dog. Renal lesions on the entire section were graded, based on the World Health Organization (WHO) grading system (1992).

Lesions graded included: cysts, interstitial fibrosis, glomerulosclerosis, amount of inflammation, and patterns of glomerulosclerosis (i.e., distribution of sclerotic glomeruli in the outer cortex, inner cortex, or in proximity to a medullary ray).

Cysts were histologically characterized based on epithelial cell lining of dilated tubules. It is thought that cysts develop as a result of an abnormality in cell differentiation and enlarge as the epithelial cells actively secrete fluid. They may also be due to the retention of urine in constricted nephron segments.

Interstitial fibrosis was characterized by the presence of ECM resulting in net collagen accumulation in the interstitium.

Glomerulosclerosis was histologically characterized by increased mesangial matrix, and adhesion of the capillary tuft to the urinary capsule.

The presence of leukocytes throughout the section determined the incidence of inflammation (Cotran, Kumar et al. 1999). This system allowed determination of the incidence and severity of the lesions and quantification of the pattern of sclerosis in the kidney.

From each slide, fifty (50) glomeruli were selected and examined based on their location, alternating between outer cortex, inner cortex, inner medullary ray and outer ray; approximately twelve (12) glomeruli in each location were evaluated per slide (Figure 3).

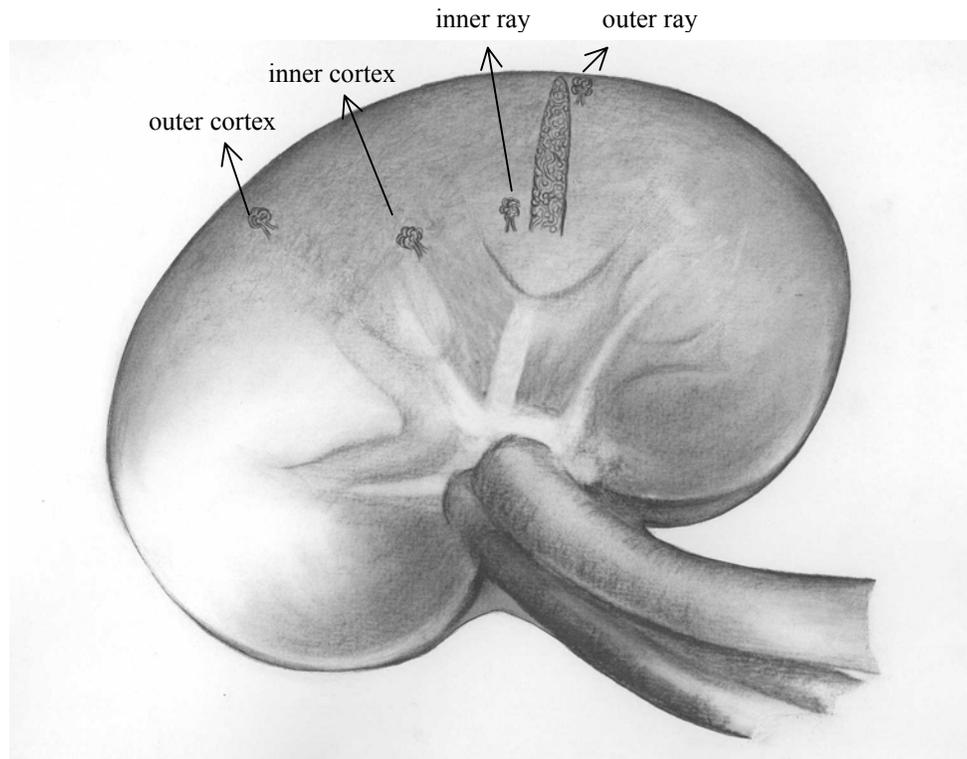


Figure 3. Sketch of a kidney, demonstrating the four glomerular locations examined in each slide.

Each selected glomerulus was graded based on four characteristics chosen to indicate the distribution and amount of collagen in and around the glomerulus: thickness of the urinary (Bowman's) capsule, thickness of mesangium, sclerosis (adhesion of tuft to the urinary capsule), and capillary bed pattern (i.e., whether the capillaries were evenly spread or divided into sections, usually as a result of thickening of the mesangial matrix). Each of the first three characteristics were scored on a scale of 0 to 5, with 0 representing no pathological change and 5 representing severe pathological changes (Table 1 and Figure 1).

Glomerulosclerosis Scoring and Glomerular Characterization

Scoring System	Characterization of Glomerulus		
Mild Sclerosis (Grade 0 or 1)	Focal capillary adhesions to urinary capsule	Capillary beds remain intact	
Moderate Sclerosis (Grade 2 or 3)	Adhesions to urinary capsule	Ballooning of peripheral capillaries; mild bed collapse	Mild thickened mesangium
Severe Sclerosis (Grade 4 or 5)	Capillary tuft occlusion; segmental tuft collapse	Inflammatory cells commonly present	Diffuse thickened mesangium

Table 1. Glomerulosclerosis was graded on the basis of WHO classification, with glomeruli ranging from minimally affected to severely affected.

Figure 4. Grading of glomerular sclerotic lesions (H&E).



After initial grading and review of the specimens, selected sections from representative dogs were identified for further evaluation by immunohistochemical (IHC) staining on the basis of age, sex, and severity of glomerulosclerosis. Males and females were grouped according to their age—those less than 7 years and those older than 7 years. Twenty (20) kidney samples from each of the male groups, fourteen (14) kidney samples from the female group under 7 years, and 20 kidney samples from the female group over 7 years were selected according to the severity of sclerosis (little to mild sclerosis or severe sclerosis), for further study (Appendix, Table 1a).

Statistical Analysis

We statistically analyzed the effects of age, sex, and location, as well as their interactions, on the severity and distribution of renal lesions in canine kidneys. Scores for renal morphologic lesions were analyzed statistically using a split-plot model, with sex and age as the whole-plot factors and location as the sub-plot factor, such that location of the glomerulus was compared to sex and age within each dog and between dogs. Data, as seen in the Results section, were analyzed with a mixed model ANOVA of the SAS system. Random effects, or factors that were chosen at random from a pool, included dogs, slides within a dog, glomeruli within a slide, and observations within a glomerulus; fixed effects, or predetermined factors, included sex, age, and location.

Immunohistochemistry

IHC staining of glomeruli was performed on selected sections using rabbit-derived antibodies to mammalian collagen types III-V (Polysciences, Inc., Warrington, PA). Enzyme-linked immunoabsorbant assay (ELISA) showed less than 1% cross-reactivity against other types of collagen (i.e., there was only 1% incorrect binding of anti-collagen antibodies to a collagen type being tested). Because cross-reactivity between rabbits and dogs had not been tested, it was necessary to establish the general cross-reactivity of the rabbit-derived antibodies, as well as determine the optimal dilution of the primary antibody. Preliminary IHC staining using tissues of several different species, including dog, rat, cat, mouse, and horse were performed on paraffin-embedded tissues at concentrations of 1:50 to 1:200. It was determined that a 1:50 dilution provided the optimal reaction.

Using a Leica Autostainer XL, deparaffinization of each slide was completed with two 10-minute washes in xylene. Graded rehydration of the sections were completed with the following protocol:

- 2 3-minute washes in 100% ethanol
- 3-minute wash in 95% ethanol
- 3-minute wash in 80% ethanol
- 5-minute wash in distilled H₂O
- 3-minute wash in distilled H₂O

Antigen retrieval was performed in order to reverse the formalin-mediated chemical modification of proteins within the kidneys (Fredenburgh, Myers et al. 2001). This process was completed by soaking the sections in a water bath at 95°F for thirty (30) minutes, followed by cooling at room temperature for twenty (20) minutes. The sections were then washed in two changes of distilled water, first for 5 minutes and then 3 minutes.

After encircling the kidney section with a PAP pen, IHC staining was performed by an Optimax Plus with the following protocol. Each step, except the last two, was followed by a buffer (1X Optimax) rinse:

- 20-minutes in 3% hydrogen peroxide (H₂O₂)

- 10-minute normal goat protein block (to reduce nonspecific binding of the primary or secondary antibody to the collagen)
- 30-minutes of primary antibody (rabbit anti-collagen)
- 20-minute anti-goat link (to bind the primary antibody to the collagen)
- 20-minute horseradish-peroxidase (HRP) label (see below)
- 5-minute 3,3' diaminobenzidine (DAB) chromagen
- 2-minute hematoxylin (a counter stain in order to differentiate between antibody and background tissue)
- Rinse with distilled H₂O

After washing in 95% ethanol, the stained kidney sections were rinsed twice in absolute ethanol followed by three washes in xylene. The specimens were then coverslipped.

HRP enzyme was selected to catalyze the reduction of H₂O₂ to H₂O and oxygen. In the presence of H₂O₂, DAB is oxidized by HRP, resulting in the polymerization of DAB molecules and the development of a dark brown precipitate. This allowed detection of the primary antibody, which was bound to the specific collagen type, through the appearance of a dark brown color (Fredenburgh, Myers et al. 2001). Therefore, the dark brown portion of the glomerulus represented that portion where type IV collagen was present in the glomerulus.

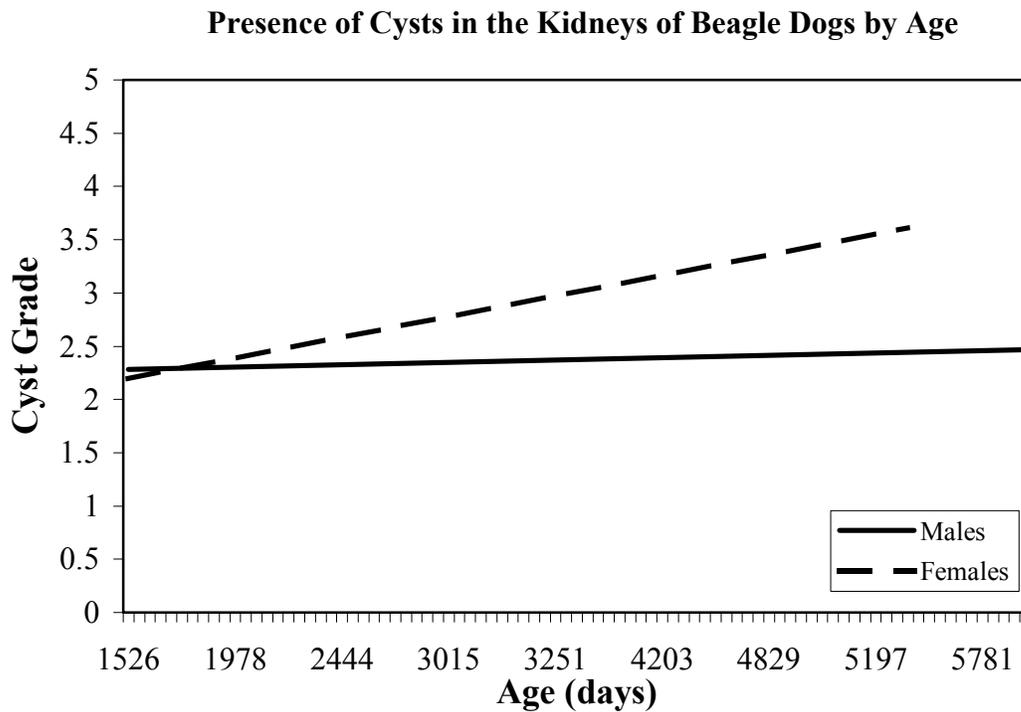
To determine percentage of collagen in a glomerulus, two slides from each selected dog were prepared. Glomeruli on each slide were again selected on the basis of location, such that two glomeruli from each location were evaluated. Using a Vanox-T light microscope (870x) with a camera attached through a serial port, images of glomeruli stained for type IV collagen were digitized with Flashbus FBG (v. 4.2) for quantification by an EV700 Gateway computer. The dark brown color of the glomerulus was expressed as the percent of collagen in a glomerulus through SCION, an NIH imaging program. Smaller glomeruli in some sections were not included in data for analysis; they were considered to be fetal glomeruli, which may be retained in the kidneys of Beagles after maturation. Such fetal glomeruli do not contain the same α -chains as mature glomeruli. Collagen types III and V were also tested for comparative purposes by visual evaluation.

Results

Renal Lesions

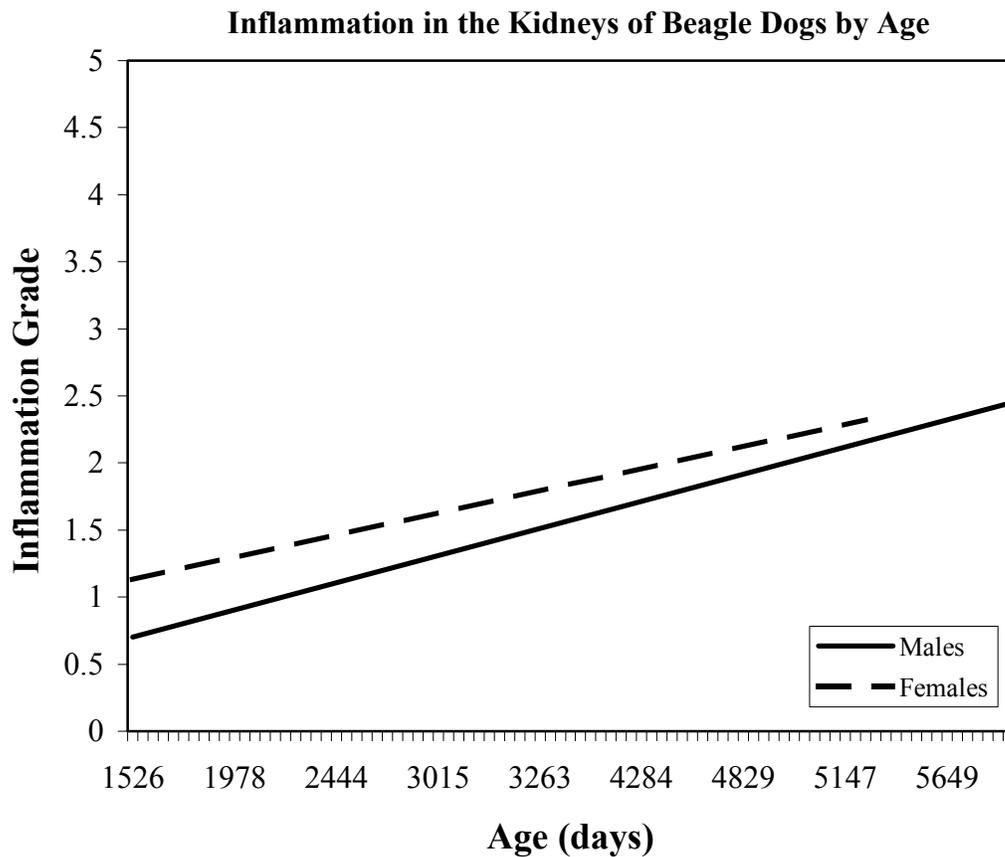
Visual inspection of data from the WHO classification of the kidneys of aging Beagle dogs collected for cysts, inflammation, and fibrosis showed the following trends:

- In females, as age increases, the presence of cysts increases. Females also develop cysts at a higher rate than male dogs (i.e., at the same age, female Beagle dogs will have more cysts present in the kidneys than males) (Graph 2),



Graph 2. Female Beagle dogs develop cysts in the kidney at a higher rate than male dogs. As the age of a female increases, the incidence of cysts increases.

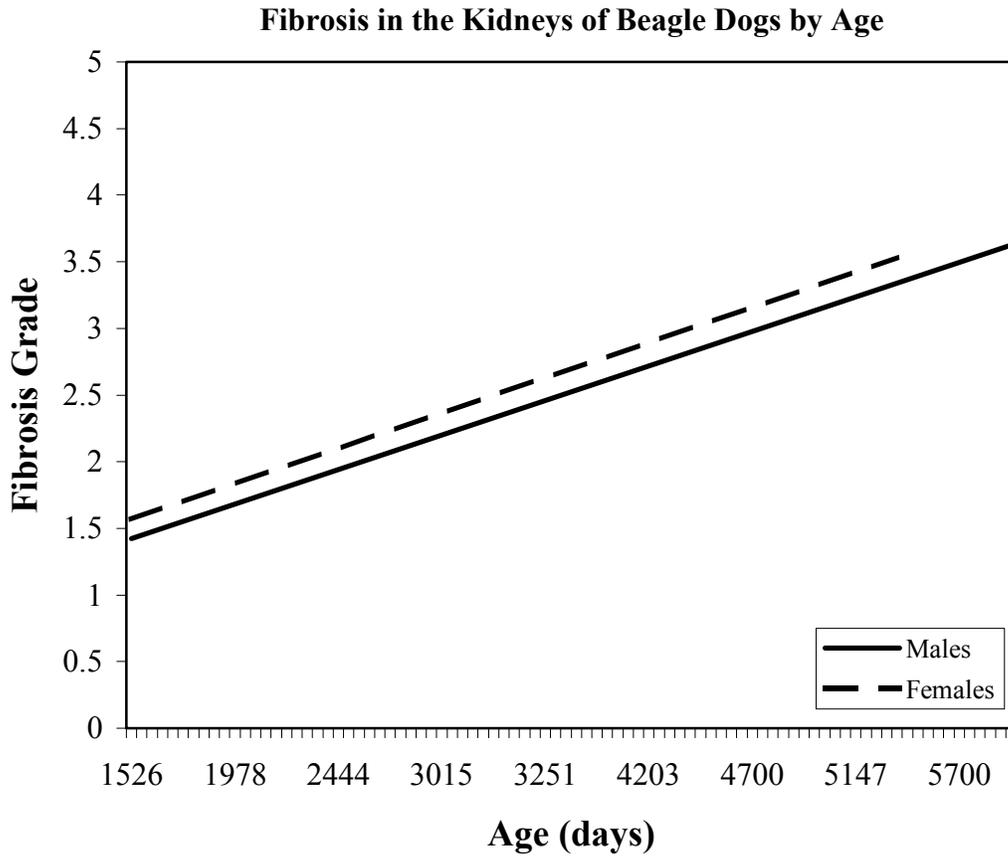
- Inflammation in Beagle dog kidneys increases with age at nearly the same rate in each sex (Graph 3), and



Graph 3. The incidence of inflammation in the kidney increases with age in both male and female Beagle dogs.

- Both male and female Beagle dogs undergo increasing fibrosis with age and this increase occurs at the same rate in both sexes (Graph 4).

Although this was not statistically tested, it appears that females consistently show a higher incidence of cysts, inflammation, and fibrosis.



Graph 4. Fibrosis in the interstitium of the kidney increases in both male and female Beagle dogs with age.

Glomerulosclerosis

Using the WHO classification system as the basis of glomerulosclerosis characterization (Materials and Methods), I initially calculated the glomerulosclerosis grade for every dog in the study by taking the average of all locations. The WHO average grade for each dog shows the increasing severity of glomerulosclerosis with age (Table 2).

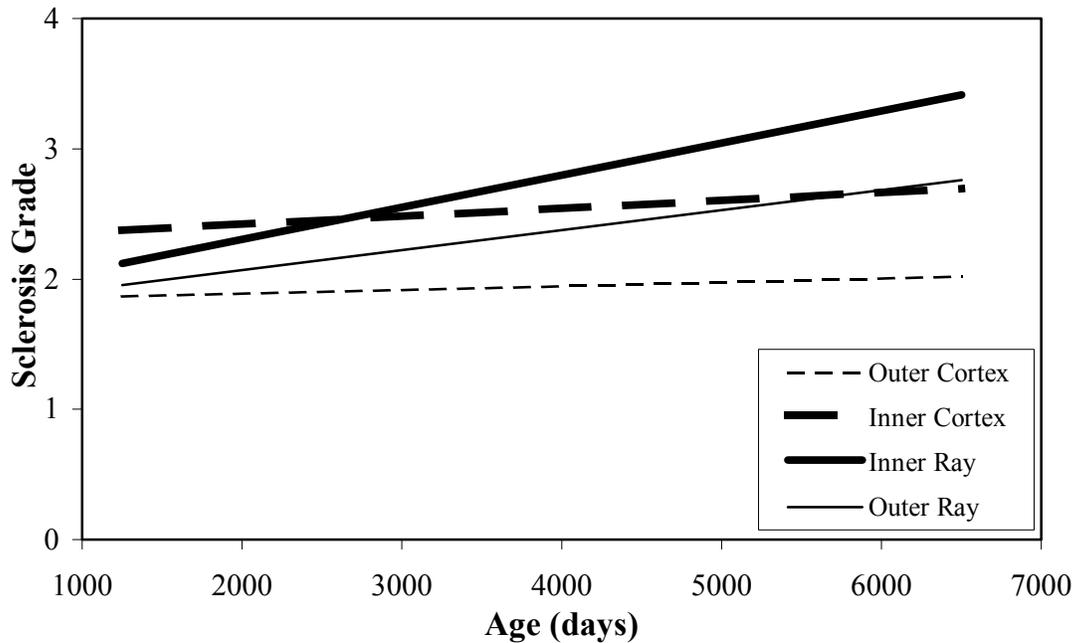
	under 7 years			over 7 years		
	Mild	Moderate	Severe	Mild	Moderate	Severe
Males	8	12	0	11	46	7
Females	5	9	0	12	41	8
Total	13	21	0	23	87	15

Table 2. There are no dogs under 7 years categorized as severely sclerotic vs. 15 dogs over 7 years. Many dogs over 7 years have an average sclerosis that can be considered moderate compared to only 21 dogs under 7 years.

In order to statistically test this observation, we first used Simple Linear Regression (SLR) showed a significant difference between males and females in the outer ray and outer cortex ($p_{\text{outer ray}} < 0.01$; $p_{\text{outer cortex}} < 0.01$), such that females show a higher incidence of glomerulosclerosis than males in the outermost region of the kidney.

However, regression may not be the best model for this experimental design as it implies that the response variable (sclerosis) is dependent on only one variable and does not include variability from dog-to-dog, glomerulus-to-glomerulus within one location within a slide, or slide-to-slide within one dog. Analyzing the data using the more appropriate split-plot model (which accounts for variability among dogs, glomeruli, and slides) showed no significant effect of sex ($p=0.128$), in contrast to the SLR analysis. There was an effect of age ($p < 0.01$) and location ($p < 0.01$), such that as age increases, glomerulosclerosis increases, with these sclerotic changes occurring at a different rate between medullary rays and the cortical labyrinth (Graph 5). Glomeruli in proximity to the medullary rays undergo significant sclerosis due to age ($p_{\text{inner ray}} < 0.01$; $p_{\text{outerray}} < 0.01$), whereas those in the cortex (i.e. not in proximity to a medullary ray) do not ($p_{\text{inner cortex}} = 0.13$; $p_{\text{outer cortex}} = 0.48$). There is also a significant difference between the glomerulosclerosis grades of each location ($p < 0.01$).

Glomerulosclerosis of Beagle Dogs by Age



Graph 5. Glomeruli in the inner and outer cortex do not significantly become more sclerotic with age; however, those near medullary rays do. Glomeruli in the inner cortex and inner ray consistently scored higher than glomeruli in the outer cortex and outer ray.

We determined the following equations predict glomerulosclerosis in the kidney of a Beagle dog:

Predictions of glomerulosclerosis by glomerular location in Beagle Dog kidneys:

$$\begin{aligned} \text{Outer cortex} &= 1.8288 + 0.000029(\text{age in days}) \\ \text{Inner cortex} &= 2.2975 + 0.000061(\text{age in days}) \\ \text{Inner ray} &= 1.8145 + 0.000246(\text{age in days}) \\ \text{Outer ray} &= 1.7628 + 0.000153(\text{age in days}) \end{aligned}$$

Renal Function as Assessed by BUN

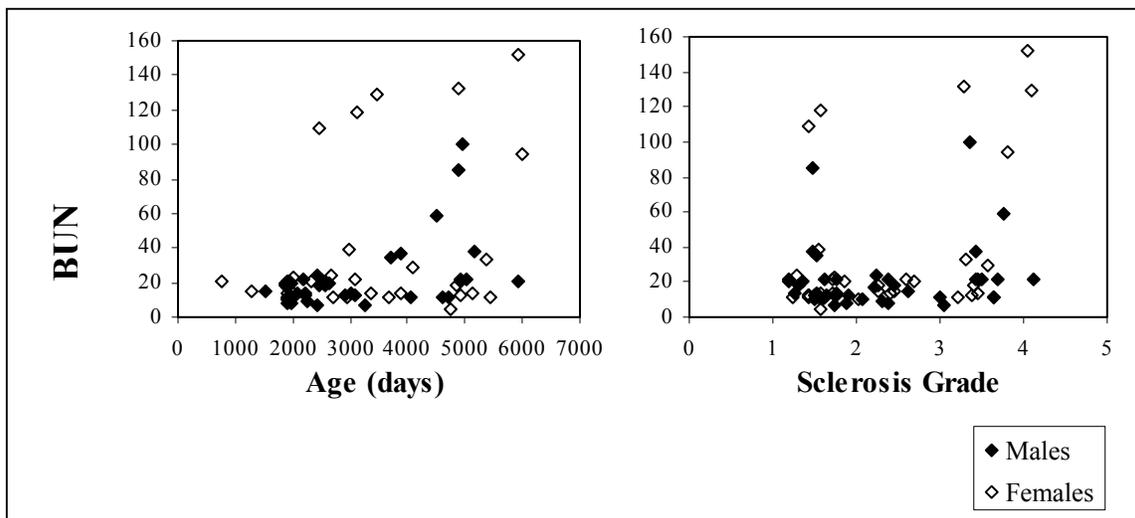
Upon obtaining functional data, including BUN, of the dogs chosen for IHC, showed that neither age nor glomerulosclerosis explain a significant effect of the variation in BUN ($r^2_{\text{age}}=0.15$; $r^2_{\text{glomerulosclerosis}}=0.11$).

Mean BUN (prior to death) of Beagle dogs by Age and Sex

	under 7 years		over 7 years	
	Mean	Std Err	Mean	Std Err
Males	14.6	5.56	37.2	43.03
Females	35.1	25.1	44.8	49.16

Table 3. While the value of BUN increases in both sexes from those under 7 years to those over 7 years, it does not appear to be significant. It should be noted that BUN of females in both age groups is elevated and in males, it is elevated only in those over 7 years.

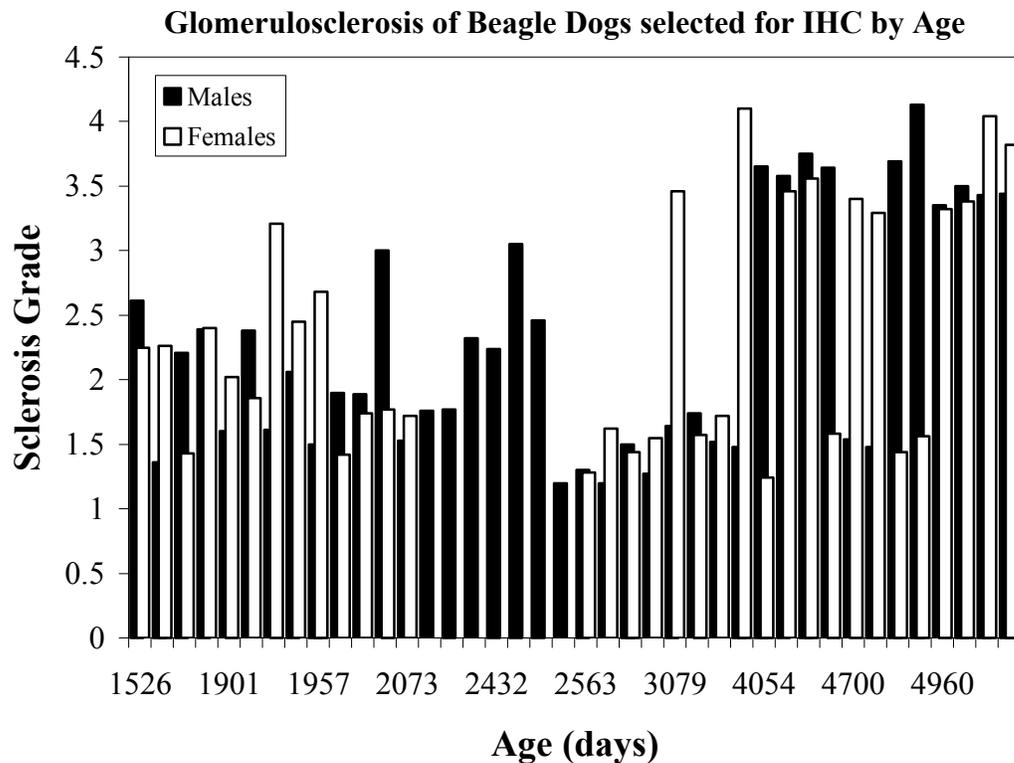
BUN (prior to death) of Beagle dogs by Age and Glomerulosclerosis



Graph 6. In both sexes, there is no correlation of age and BUN or sclerosis and BUN. Consequently, BUN values of a Beagle dog cannot be predicted by knowledge of either the age of the animal or sclerosis grade upon biopsy.

Collagen Deposition

The split-plot model was again used for analysis of type IV collagen percentage of the glomerulus. The ages of the dogs ranged from 762 days to 6011 days.



Graph 7. Females generally had higher average sclerosis grades of dogs selected for IHC. This trend is more evident in dogs over 7 years (2555 days). There were fewer females under 7 years selected for IHC as that population of dogs numbered only 14 (vs. 20 male dogs).

The mean collagen deposition of each location, as determined by SCION, was calculated for both age groups by sex (Table 4).

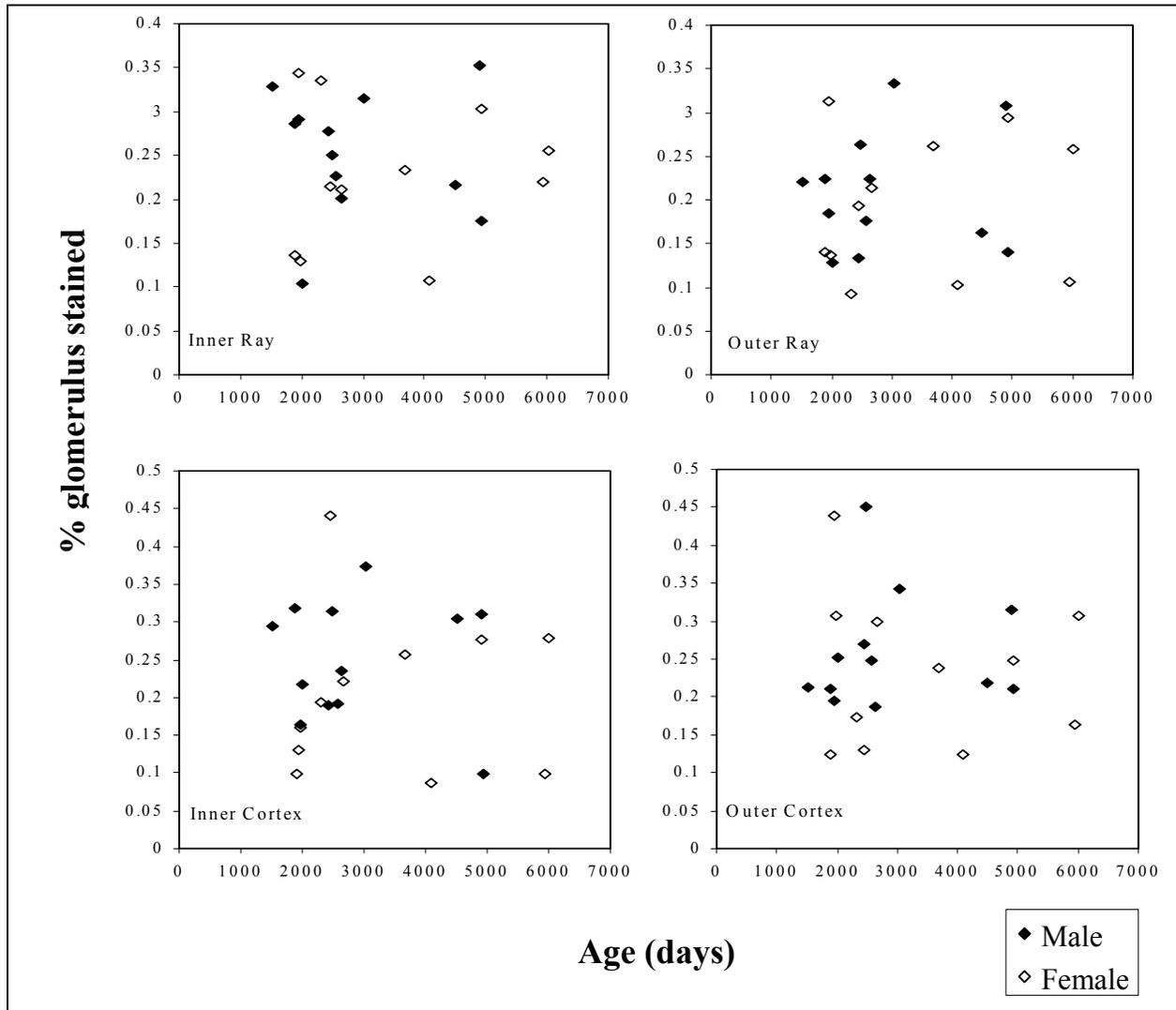
**Mean Type IV Collagen Deposition in Beagle Dog
Glomeruli by Age and Sex**

under 7 years				over 7 years			
Sex	Location	Mean	Std Err	Sex	Location	Mean	Std Err
Males	Inner Ray	0.256	0.032	Males	Inner Ray	0.247	0.028
	Outer Ray	0.192	0.022		Outer Ray	0.224	0.033
	Inner Cortex	0.25	0.028		Inner Cortex	0.252	0.04
	Outer Cortex	0.265	0.039		Outer Cortex	0.253	0.025
Females	Inner Ray	0.232	0.046	Females	Inner Ray	0.222	0.026
	Outer Ray	0.175	0.038		Outer Ray	0.256	0.034
	Inner Cortex	0.205	0.061		Inner Cortex	0.203	0.036
	Outer Cortex	0.234	0.061		Outer Cortex	0.23	0.03

Table 4. In both sexes, the mean collagen deposition in the inner ray and outer cortex between those under 7 years and those over 7 years actually decreased. Glomeruli in the outer ray and inner cortex show a minimal increase in type IV collagen deposition.

No significant effect of sex, age, or location was seen ($p_{\text{sex}}=0.7029$; $p_{\text{age}}=0.819$; $p_{\text{location}}=0.5932$) (Graph 8). Therefore, there was no significant increase in type IV collagen due to either age, sex, or glomerular location.

Type IV Collagen Deposition in Beagle Dog Kidneys by Age and Glomerular Location

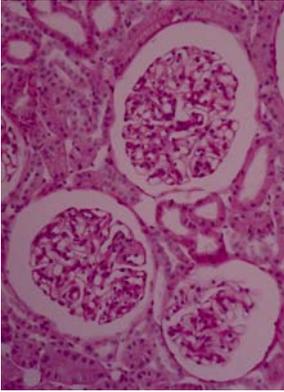


Graph 8. In both sexes and all locations, there is no correlation between the age of the Beagle dog and percent of type IV collagen present in the glomerulus. Consequently, there is no increase in type IV collagen by sex or as age increases.

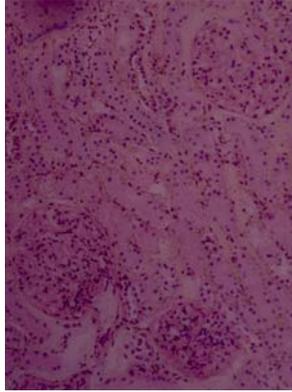
Visual evaluation of types III and V collagen showed distinct differences in the staining pattern, and therefore collagen deposition (Figure 5).

PAS and IHC Staining Between Dogs of Different Ages

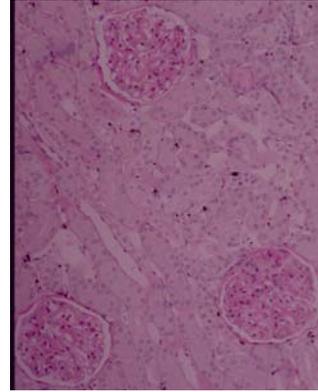
PAS



a) Dog 10429, 1884 days.

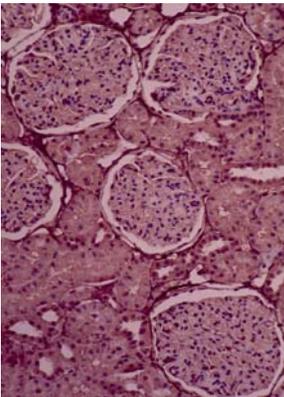


b) Dog 6577, 2436 days.

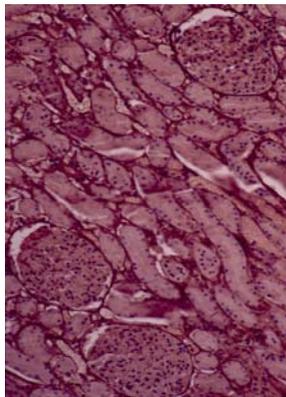


c) Dog 10803, 2633 days.

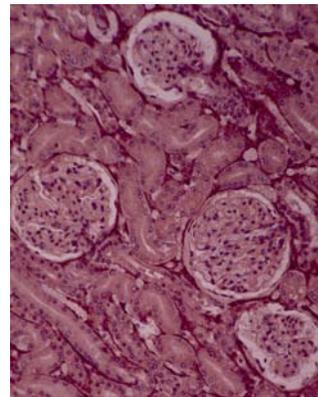
Type III collagen



d) Dog 10429.

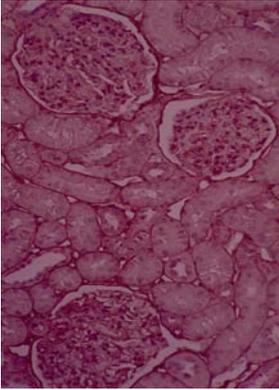


e) Dog 6577.

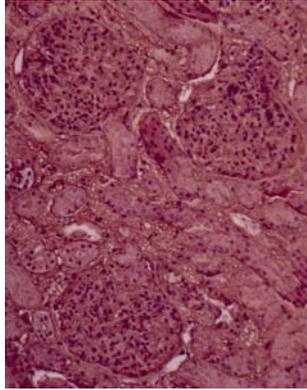


f) Dog 10803.

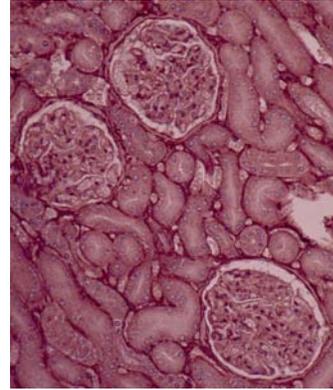
Type IV collagen



g) Dog 10429.

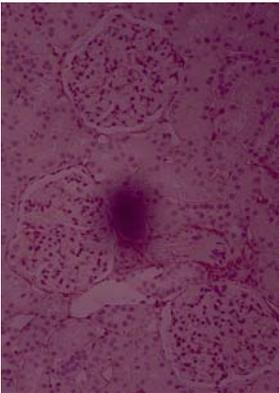


h) Dog 6577.

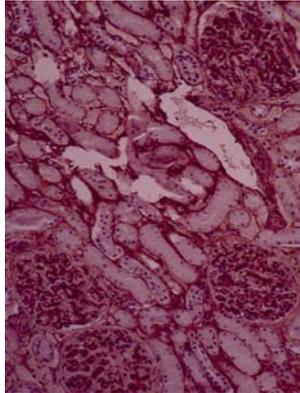


i) Dog 10803.

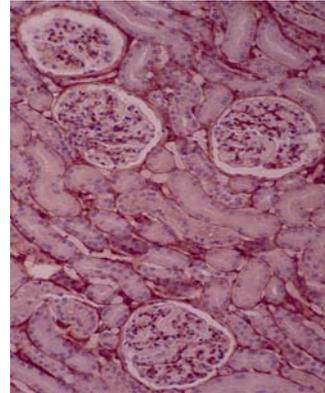
Type V collagen



j) Dog 10429.



k) Dog 6577.



l) Dog 10803.

Figure 5. In comparing the three types of collagen, it appears that type III collagen is heavily concentrated in membranes of the tubules, while there is less type V collagen present throughout the section. Type IV collagen appears more localized in the glomeruli vs. the interstitium.

Discussion

Renal Lesions and Glomerulosclerosis

This thesis project was an evaluation of the effects of aging on the canine kidneys. This study provides a baseline for assessment of the aging process of the kidney, which can be applied in long-term studies. It is important to consider structural and functional changes the kidney may undergo due to aging alone, and to consider them separately from changes that result from disease. I found several trends of age-related changes in the kidney:

- Females Beagle dogs are more prone to develop cysts associated with increasing age;
- Inflammation and fibrosis increases with age at nearly the same rate in males and females, with fibrosis occurring more frequently throughout life;
- The incidence and severity of glomerulosclerosis increases with age and it is essential to consider the location of the glomerulus being studied, especially when examining glomeruli in proximity to medullary rays;
- BUN alone is not an accurate measurement of kidney function nor a good predictor of age-related changes in either sex.

One interesting trend is seen with juxtamedullary glomeruli, or those in the inner cortex and inner rays (Graph 5). These glomeruli consistently received higher WHO classification scores than glomeruli in either the outer cortex or outer rays, demonstrating that these glomeruli were most affected by glomerulosclerosis *regardless of age*. Studies have shown that renal blood flow to the cortex accounts for 98 to 99 percent of total renal blood flow (Guyton and Hall 1998). I speculate that the higher glomerulosclerotic score of juxtamedullary glomeruli may be due to the fact that these glomeruli become more perfused upon insult to the kidney, in accordance with the hyperfiltration hypothesis. Therefore, juxtamedullary glomeruli undergo glomerulosclerosis while blood is shunted away from cortical glomeruli. Furthermore, experimental data measuring intra-renal blood flow through implanted optical fibers has shown that blood flow in the deep cortex is generally well autoregulated, while inner and outer medullary blood flow is poorly autoregulated. A study by Mattson et al. (1993) demonstrated that blood in the post-glomerular circulation of deep juxtamedullary glomeruli may be redistributed as renal perfusion pressure is decreased, such as in the case of hydropenia (water deprivation) (Mattson, Lu et al. 1993).

It is normal to have some level of urea in the blood, but to exceed this range, which varies between species, signifies a problem in the ability of the kidneys to remove nitrogenous wastes from the blood. Generally speaking, BUN does increase with age, presumably as a result of deteriorating kidney function. However, the results of this study suggest that neither age nor glomerulosclerosis alone explains a significant part of the variation in BUN, as these two factors are collinear and therefore cannot be separated. Furthermore, based on the analysis of BUN prior to death, functional changes do not occur in every dog at the same rate (Graph 6).

While altered function may precede structural damage to the organ, one cannot predict the degree of glomerulosclerosis based strictly on BUN (Lerman and Rodriguez-Porcel 2001). It is essential to use other clinical tests, including creatinine and proteinuria, in order to accurately assess the functional capability of the kidneys. Only by looking at the results of these other tests in concert with BUN will the correct conclusion of the extent of renal failure be deduced. These procedures are also important in the monitoring of renal disease.

Collagen Deposition

In regard to the data on type IV collagen deposition, these results indicate that there is no accumulation of type IV collagen within dog glomeruli due to aging. As with the grading portion of the analysis, no significant difference was found between males and females. Unlike the grading of glomerulosclerosis, no type IV collagen accumulation due to the location of glomeruli within the kidney was seen (Graph 8).

These results differ from that of Striker et al. (1997), who found an accumulation of type IV collagen characterized progressive glomerular diseases in humans with diabetes, renal cancer, or MGN (Striker, Esposito et al. 1997). The discrepancy between the Striker study and this study may be due to one or several factors:

- technique—this study used IHC analysis whereas the Striker study used mRNA analysis,
- diseased state—the kidneys of the subjects in the Striker study were undergoing structural and functional changes primarily as a result of disease, whereas the dogs in this study did not have chronic disease,
- or, species differences—Beagle dogs vs. humans. The dogs in this study were fed the same food and drank from the same water source. They were also exposed to the same environmental conditions. The humans in the Striker et al. study did not eat the same food, drink the same water, and live in the exact same location.

Finally, it is possible that there may be a biochemical alteration of type IV collagen with increasing age. Chemical analysis of diabetic glomeruli in a study by Churg and Dachs (1975) showed an excess of hydroxyproline in diabetic glomeruli. In comparing this to a study by Bonting et al. where it was determined that normal glomeruli contain 1% of the amino acid hydroxyproline, “abnormal glomeruli contained considerably more of this amino acid.” These authors concluded that the collagen present in the diseased glomeruli is in a different form from the collagen of loose connective tissue (Churg and Dachs 1975).

A study by Brownlee (1995) showed that advanced glycation end-products (AGEs) accumulate in tissues as a function of age and sugar concentration. AGEs may produce pathological changes through several mechanisms. For instance, AGE formation in type IV collagen inhibits lateral association by interfering with the binding of the NC1 domain to the 7S domain, such that the collagen cannot form a normal network-like structure. AGE formation on ECM also has been shown to interfere with matrix-cell interactions and decrease the ability of laminin to bind type IV collagen. High-affinity receptors for

AGEs have been identified on monocytes and macrophages (Brownlee 1995). Therefore, it should not be considered abnormal to see inflammatory cells at the site of AGE deposition on type IV collagen, and this phenomenon would then increase with age. Therefore, to more thoroughly investigate the correlation of inflammation and glomerulosclerosis in the absence of kidney disease may provide evidence of biochemical changes to type IV collagen.

The tests for cross-reactivity (see Materials and Methods) showed that rabbit-derived type IV collagen antibody does, in fact, recognize canine type IV collagen. It is therefore possible that these antibodies bind the molecule around the *Gly-Xaa-Yaa* repeats. If this were not the case, the specificity of the reaction would be affected. AGE accumulation on the ends of the molecule thus cannot interfere with recognition of canine type IV collagen because the recognition site is in the domain that is not biochemically altered.

Upon examining the possibilities of other types of collagen being deposited, no significant accumulation of either type III or type V collagen appeared in the glomeruli of aged dogs. Type III collagen did appear more heavily concentrated in the interstitium than did type IV collagen, while there appeared to be less type V collagen deposition overall than either type III or type IV collagen. Further studies are needed to clarify this.

Conclusions drawn from this study may provide a basis for extrapolation to other species, including man. Since Beagles serve as the primary animal model in chronic toxicological studies, this study also has relevance in respect to age-related changes that may occur in the kidneys of animal models in terms of structure and function, possibly affecting toxicology study analysis.

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Appendix.

Distribution of Kidney Sections Selected for Immunohistochemistry

Sex	Sclerosis	under 7 years	over 7 years
Males	Little-Mild	10	10
	Severe	10	10
Females	Little-Mild	7	10
	Severe	7	10

Table 1. After averaging all graded glomeruli within a dog, we selected representative dogs for IHC staining based on severity of sclerosis. Twenty (20) kidney samples from the male group under 7 years and the group over 7 years, fourteen (14) kidney samples from the female group under 7 years, and 20 kidney samples from the female group over 7 years were selected.