URINE PROTEIN ANALYSIS AND CORRELATION OF URINARY BIOMARKERS WITH RENAL DISEASE PROGRESSION IN DOGS WITH X-LINKED HEREDITARY NEPHROPATHY

A Dissertation

by

MARY B. NABITY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2010

Major Subject: Veterinary Pathology

Urine Protein Analysis and Correlation of Urinary Biomarkers with Renal Disease Progression in Dogs with X-Linked Hereditary Nephropathy

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ABSTRACT

Urine Protein Analysis and Correlation of Urinary Biomarkers with Renal Disease Progression in Dogs with X-Linked Hereditary Nephropathy. (December 2010)

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Chronic kidney disease (CKD) is a major cause of illness in dogs, and it is commonly caused by glomerular diseases that result in proteinuria and a progressive decline in renal function. Despite the importance of glomerular lesions, tubulointerstitial fibrosis identified by histologic evaluation of renal biopsies correlates best with renal function. However, performing a renal biopsy is invasive. Most current non-invasive tests for renal function lack adequate sensitivity and specificity for renal disease. Proteinuria can be both a sensitive and specific marker for renal damage. However, its evaluation in veterinary medicine beyond determination of the magnitude of proteinuria (e.g., urine protein:creatinine ratio (UPC)) is limited. Therefore, in this report, further evaluation of the UPC was performed to aid in the monitoring of renal disease progression and response to treatment. In addition, qualitative evaluation of proteinuria was performed in dogs with progressive CKD in order to identify better non-invasive markers for tubulointerstitial injury.

The day-to-day variability of the UPC was determined utilizing data obtained from female dogs that are carriers for X-linked hereditary nephropathy (XLHN). Despite

an unchanging magnitude of proteinuria in these dogs, substantial variation in their UPC was observed. Using these results, guidelines were suggested to help assess whether disease progression or treatment leads to a significant change in UPC.

Qualitative characterization of proteinuria in dogs with CKD was performed using urine from male dogs affected with XLHN, and results were correlated with clinical and histologic findings concerning renal function and damage. The two discovery proteomic techniques utilized (chromatographic chip array and two-dimensional gel electrophoresis) revealed several proteins that have not previously been implicated as markers for canine CKD, providing a basis for future studies. Specific assays for urinary biomarkers of renal injury were used to serially evaluate renal function in these dogs. All proteins evaluated proved to be sensitive markers for renal damage. However, only retinol binding protein provided clear evidence for renal disease progression. These results will provide the foundation for future studies aimed at monitoring urinary biomarkers in dogs with CKD, which will ultimately help veterinarians better diagnose and monitor proteinuric renal disease.

DEDICATION

For Craig and Gwenny

ACKNOWLEDGEMENTS

Throughout this project and my time at Texas A&M, I have been fortunate to work with some truly inspirational people. In particular, I would like to thank my mentor and co-chair, Dr. George Lees, for his patience, guidance, and selfless promotion of my work and career. He is one of the most conscientious people I have met, and I feel fortunate to be able to call him my mentor and to have the opportunity to continue to work with him in the field of renal pathology.

I would also like to extend my sincere thanks to the remaining members of my committee: my other co-chair, Dr. Fred Clubb, for always being willing to help and for enthusiastically agreeing to join my committee fairly late in the process; Dr. Brian Berridge, who helped spur on the initial discussions regarding this project and who has always provided sound and sage advice; Dr. May Boggess, who provided the most excellent statistical expertise I could ask for; and Dr. Jörg Steiner, who welcomed me into his laboratory space to carry out much of my project. His help along with the expertise of the entire Gastrointestinal Laboratory was invaluable, and in particular, I would like to thank Dr. Jan Suchodolski for always offering suggestions for the setup and analysis of the various components of my project.

In addition to the GI Laboratory, I had several other laboratory "homes" throughout the last several years, including the laboratory of Dr. Shashi Ramaiah, who also served as my co-chair for several years; the Protein Chemistry Laboratory, headed by Dr. Larry Dangott, who helped to make the two-dimensional gel work enjoyable; and

the canine genetics laboratory of Dr. Keith Murphy, where I met many fun and helpful graduate students over the years. The completion of this project would not have been possible without these various laboratories extending their hospitality.

Of course, this project depended on the careful maintenance of the dog colony as well as proper collection and storage of samples obtained from the dogs over a period of 8 years. Mary Sanders and the many student workers who have worked with her over the years have done a tremendous job ensuring that everything ran smoothly from day to day. The dogs always helped to provide an adorable retreat, and it was encouraging to see them maintain a cheery disposition throughout the studies, even when their disease started to get the best of them.

Thanks also to my clinical pathology colleagues for allowing me time away from clinics to complete the laboratory portions of this project. In addition, I would like to extend my gratitude to the Morris Animal Foundation for providing me with my first grant that helped to fund a large portion of these studies.

Finally, I thank my husband, Craig, for coming into my life at the perfect time—he is one of the main reasons I have chosen the path I am on today. He has provided never-ending support as I have continued to further my educational training, and he has endured my long hours and occasional irritable moods with an encouraging sense of humor and selflessness. In addition, his professional and personal guidance have been invaluable as I prepare for an academic career.

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INTRODUCTION

Overview of canine chronic kidney disease

Chronic kidney disease (CKD) is a major cause of morbidity and mortality in dogs, and it can be due to primary renal diseases or secondary to a variety of systemic diseases.[1] Structural and functional evidence of kidney damage is prevalent, even in apparently clinically healthy dogs, and prevalence of disease increases with age, approaching 50-90% in some studies.[1-3] While evidence of kidney damage is common, progressive CKD is less commonly recognized. It is generally accepted that CKD is present in approximately 15% of dogs older than 10 years of age, and that it is responsible for 3-5% of all deaths in dogs.

CKD may be initiated by glomerular damage, tubulointerstitial damage, or both. Historically, canine CKD was thought to arise primarily due to tubulointerstitial damage, but as more data about renal disease becomes available and as veterinarians have become more astute in detecting CKD earlier during the disease process, it has become clear that glomerular disease is a significant, if not predominant cause of CKD.[3,4] Regardless of the cause, renal function correlates best with renal tubulointerstitial changes such as interstitial fibrosis, tubular degeneration and atrophy, peritubular capillary loss, and ultimately, destruction of functional nephrons.[5]

This dissertation follows the style of Journal of Veterinary Internal Medicine.

Progression of renal lesions to CKD ultimately depends on whether continued nephron loss occurs either as a result of or after the initiating injury. Disease progression involves a complex interplay of numerous pathophysiologic mechanisms that involve all components of the kidney. For instance, when enough nephron loss has occurred to impair renal function, hyperfiltration of remaining nephrons occurs due to glomerular capillary hypertension and hypertrophy, which can have a number of harmful downstream effects on the affected glomeruli and tubules, including proteinuria, hypoxia, and stimulation of inflammatory pathways.[6,7] This self-perpetuation of nephron damage results in renal disease progression even after the initiating cause has been removed. In addition, proteinuria due to glomerular damage leads to production of inflammatory, vasoactive, and fibrogenic mediators by the renal tubules.[8]

Current non-invasive methods for detecting renal disease

While tubulointerstitial lesions observed in renal tissue correlate best with the degree of renal function, renal biopsy is a relatively invasive technique that is not commonly performed in veterinary medicine. Therefore, non-invasive tests of renal function are more often utilized. The gold standard for global renal function is estimation of the glomerular filtration rate (GFR) using clearance methods to determine the volume of plasma cleared of its marker substance by the kidney in a unit of time. In this way, the estimated GFR provides a measure of the ability of the kidneys to clear the blood of waste metabolites. Several endogenous substances are typically used to provide a rough indication of GFR, as described below.

Renal function and GFR can be altered by either glomerular disease or tubulointerstitial disease. It would be clinically useful for the clinician to be able to distinguish between damage to these two components. Clinical tests for monitoring both glomerular and tubulointerstitial damage are available, but they have significant limitations. Tubulointerstitial damage is routinely detected clinically by increases in serum creatinine concentration (sCr) in combination with a decreased urine specific gravity (USG). Serum creatinine is currently used to stage the severity of CKD in dogs using the IRIS (International Renal Interest Society) staging system with sCr > 1.4 mg/dl being considered azotemic in most dogs. Urine specific gravity in renal failure is typically 1.008-1.012, which is referred to as isosthenuria, and this indicates impaired concentrating ability by the tubules. However, changes in both sCr and USG are insensitive, occurring only after a substantial (65-75%) loss of nephrons.[9] They also are both affected by certain non-renal factors that can decrease their specificity. For example, creatinine production is primarily determined by the muscle mass of the patient. Therefore, measurement of serum creatinine concentration may overestimate renal function in cachectic, geriatric, and very young patients, and in dogs, breed and weight must also be considered.[10] Because of this, basing a normal versus abnormal value on a global reference interval for sCr will be inaccurate in many patients. However, while inter-individual variation can be quite large, intra-individual variation in a healthy adult animal is small over weeks to months, and even years.[11] Therefore, serial monitoring in a patient may allow detection of mild elevations in serum creatinine concentration that might be associated with significant renal disease. For USG, values

are influenced by hydration status, medications, and a variety of diseases and endogenous substances. This poor specificity is illustrated clinically, where dogs without evidence of renal disease can have a USG ranging from 1.001-1.045. By combining an increased sCr with an isosthenuric USG, specificity for renal failure rises, but even this combination is not specific for primary renal disease, exemplified by some cases of hypoadrenocorticism. Therefore, more sensitive and specific non-invasive tests of tubulointerstitial injury and function are needed.

Glomerular damage can be first detected clinically by renal proteinuria, and an increased urine protein:creatinine ratio (UPC) $\geq$ 2.0 is currently one of the best non-invasive indicators to detect the presence of glomerular disease.[12] However, patients with a mildly increased UPC of between 0.5-2.0 may either have glomerular or tubular disease. The UPC provides an indication of the magnitude of proteinuria, and in general, the higher the value the more severe or extensive the glomerular lesion. However, this is not always the case, and UPC may actually decrease with end-stage renal failure due to a decrease in the number of functioning nephrons.[12] The urine protein concentration measured for determination of UPC represents the total protein present in the urine, which is generally mostly composed of albumin, globulins, and Tamm-Horsfall protein. Urine creatinine concentration is used in the calculation of the UPC to account for the marked variation of the urine concentration that occurs in a given patient due to changes in hydration status. Monitoring the UPC can be useful to detect progression or improvement of glomerular disease, but many factors may contribute to variation of the UPC without any change in the prevailing magnitude of proteinuria. Therefore, it can be

difficult to determine whether a true increase or decrease in the magnitude of proteinuria has occurred using serial UPC measurements. It is important to note that the presence of microalbuminuria can serve as an indicator of glomerular damage that is more sensitive than an increased UPC.[13] However, its overall lack of specificity limits its use as a reliable indicator of clinically significant or progressive glomerular or tubular injury.[14,15]

Proteinuria in kidney disease

Normal urine contains only a small amount of protein, which is due to the combined efforts of both the glomeruli and the proximal renal tubules. In dogs, normal 24-hour urinary protein loss has been reported to range from 50-300 mg/day, although this estimate depends on the study population and assay used for protein measurement, since widely varying results may be obtained using different assays.[16-18] This amount of urinary protein loss typically corresponds with a UPC < 0.2.[17,18] The causes of proteinuria may be divided into pre-renal, renal, and post-renal abnormalities. Examples of pre-renal causes of proteinuria include dysproteinemias (e.g., multiple myeloma), whereas post-renal causes of proteinuria are a result of excretory pathway inflammation and/or hemorrhage (e.g., urinary tract infection). These causes of proteinuria are important to recognize and treat, but quantification of proteinuria in these situations is not helpful in assessing disease severity or prognosis. On the other hand, kidney disease in people and dogs is commonly characterized by protein loss, and quantification of proteinuria that is secondary to renal disease is clinically useful. Several studies have shown that a greater magnitude of proteinuria correlates with the severity and

progression of renal disease in dogs.[19,20] In addition, institution of therapies that reduce the magnitude of proteinuria help to slow the progression of renal disease.[21-25]

The concept behind proteinuria as an indicator of renal damage is that the glomerular filtration barrier normally excludes most proteins that are the size of albumin or larger (> 60 kDa) based on size-exclusion studies.[26] Proteins smaller than this, which are referred to as low molecular weight proteins (LMW), typically pass through the barrier, and their ease of passage depends on their size, charge, and shape. However, the proximal tubules efficiently reabsorb these LMW proteins.[27] Since larger proteins, particularly albumin, comprise the bulk of the proteins in plasma, glomerular damage can lead to massive renal protein loss, whereas tubular damage generally results in only mild proteinuria. While it is generally accepted that the glomeruli provide the main barrier to albumin filtration, debate continues as to the relative contribution of the glomerulus versus the tubules in development of albuminuria.[28]

The glomerular capillary filtration barrier

The glomerular capillary wall is composed of three major structural components: the fenestrated capillary endothelium, the glomerular basement membrane (GBM) and visceral epithelial cells called podocytes (Figure 1). These components all closely interact with one another to form a highly specialized filtration barrier that serves to retain the cellular components and the majority of plasma proteins in the blood. Normal function of this barrier depends on normal structure and function of each component as well as a normal hemodynamic steady state.[29] Alterations in any one component can lead

to altered function of the glomerular capillary barrier as a whole.[30] In addition, mesangial cells, while not in the direct line of plasma filtration, influence glomerular filtration and help to maintain both the structural and functional integrity of the glomerular capillary loop.[26,31] The downstream effects of an altered filtration barrier include clinical manifestations of proteinuria and hematuria, and in many cases, progression to end-stage renal disease. However, there are still many controversial aspects regarding the filtration barrier and its role in proteinuria, including which structural component provides the main filtration barrier for proteins and the importance of charge versus size of a protein in preventing its passage.[30] Depending on the study, anywhere from 0.06% to 3% of plasma albumin is estimated to pass through the filtration barrier.[30]

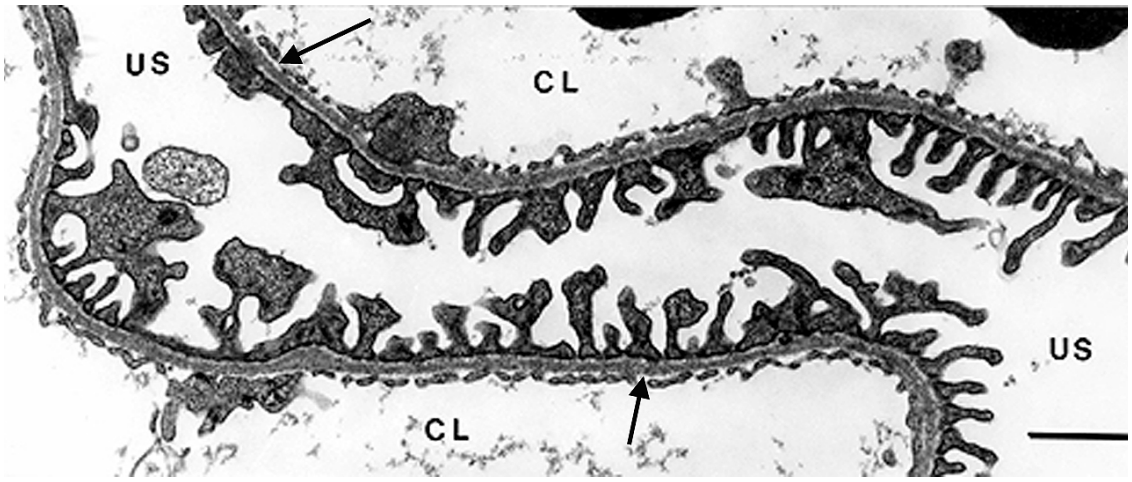

Figure 1. Transmission electron micrograph of the glomerular capillary wall of a normal dog. Endothelial cells line the capillary lumen (CL), while podocyte foot processes line the urinary space (US) aspect of the glomerular capillary wall. The basement membrane (arrows) is located between these two cells types.

Capillary endothelium

The "internal" component of the glomerular filtration barrier is the capillary endothelium. Importantly, this endothelium contains fenestrations of approximately 60-80 nm that allow for filtration of plasma through the endothelium, and these fenestrae appear to be induced and maintained by VEGF.[32] Endothelial cells are internally lined by a negatively-charged glycocalyx composed predominantly of proteoglycans, namely heparan sulfate proteoglycans.[33] This glycocalyx covers both fenestrated and non-fenestrated portions of the endothelial cell,[34] and some investigators propose that this layer should be considered as a separate and thus fourth component of the glomerular filtration barrier.[35] Certainly, studies have shown that administration of enzymes to break down the glycocalyx result in increased permeability of the glomerulus.[36]

In examples of glomerular endothelial damage, the most studied of which is preeclampsia, proteinuria and a decrease in glomerular filtration rate are seen, and in some cases the proteinuria is present in the absence of evident basement membrane and podocyte damage.[32] This highlights the importance of the endothelium in either preventing passage of albumin or in functionally altering the other barrier components through signaling mechanisms.[32]

Glomerular basement membrane

The glomerular basement membrane (GBM) is produced by both podocytes and endothelial cells.[37] The main components of the GBM are laminin, type IV collagen, nidogen/enactin, and proteoglycans, including the heparan sulfate proteoglycans agrin and perlecan.[38] In addition to being thicker than most basement membranes, a

distinguishing characteristic of the GBM is the presence of laminin-521 (α5β2γ1) and type IV collagen composed of α3, α4, and α5 chains.[38] Ultrastructurally, the GBM is characterized by three layers: the middle lamina rara densa, the sub-endothelial lamina rara interna, and sub-epithelial lamina rara externa.[39] Although some controversy exists, it is widely accepted that the GBM provides both a charge- and size-selective barrier to the passage of proteins.[40]

Diseases that are characterized by mutations of genes that encode GBM proteins illustrate the importance of the GBM in maintaining permselectivity of the glomerular filtration barrier and in renal function. For instance, people and mice with mutations of the gene encoding the β2 chain of laminin develop marked proteinuria and progressive renal disease, and in mice, the proteinuria occurs before there are any evident changes of the podocytes.[41] Similarly, dogs with mutations of genes that encode the α4 or α5 chain of type IV collagen (analogous to Alport syndrome in humans) develop early and marked proteinuria as well as progressive renal disease.[42-46] Interestingly, in people with Alport syndrome, the earliest and most striking clinical feature is hematuria rather than proteinuria. While proteinuria may be present in these patients, it is initially mild, although nephrotic syndrome is common in those patients with advanced nephropathy.[47] Because of this, type IV collagen is generally not thought to play an important role in macromolecular filtration.[48]

The molecular basis for the development of renal disease and proteinuria with disruption of GBM components is unknown. However, in patients with Alport syndrome the glomerular basement membrane is composed of α1-α2(IV) chains rather than

predominantly of α3-α4-α5(IV) chains.[49] The α1-α2(IV) chains are more susceptible to proteolysis, possibly due to fewer disulfide bonds, and this may explain disease onset and progression.[50] In addition, it is well-known that there are extensive connections that allow for signaling between the GBM components and the adjacent podocytes and endothelium.[51] An alteration of the composition of type IV collagen may alter signaling to these cells, thereby contributing to the observed proteinuria and progressive renal disease.

Podocytes

Podocytes are present along the urinary space aspect of the glomerular capillary wall. Podocytes were historically thought to have little to no capacity for regeneration or renewal; however, recent studies have identified podocyte progenitor cells within Bowman's capsule.[52] Podocytes extend cytoplasmic processes called foot processes that essentially form a covering around glomerular capillary loops. Foot processes are attached to the GBM mainly through integrins, although tetraspanins and dystroglycans appear to play a role as well.[53] Interestingly, deficiency of the tetraspanin CD151 in mice results in similar GBM ultrastructural findings as Alport syndrome.[54] The foot processes from two adjacent cells interdigitate along the capillary wall, and these processes are connected together by a unique structure called a slit diaphragm. This diaphragm forms a zipper-like shape, with pores smaller than albumin.[53] Unique proteins such as nephrin and Neph1-3 help to form the "zipper" that spans the two foot processes, but a number of other components are necessary for proper functioning of this structure, including podocin, Cd2ap, and Nck, among others.[53,55] Along the apical side of the foot processes

are podocalyxin and Glepp1, which are both proteins necessary for normal podocyte architecture and filtration.[53,56] Maintenance of the actin cytoskeleton is also crucial for proper podocyte functioning, and this is mediated in large part by α-actinin-4 and synaptopodin.[53] Mutations in many of these proteins have been shown to result in massive proteinuria in people and mice and in many cases, rapid renal disease progression is seen.[55]

With the recent discovery of the various components of the slit diaphragm and their relation to genetic proteinuric disease, primary emphasis has recently been given to the podocytes in the development of proteinuria. In addition, in most proteinuric nephropathies podocyte foot process effacement, which is the primary means for detecting podocyte damage, has been observed. However, whether effacement is a cause or consequence of proteinuria in various diseases remains debated. For example, proteinuria occurs prior to podocyte foot process effacement in experimental animal models with certain defects in each of the 3 components of the glomerular capillary wall.[51] These findings suggest that the interplay among the various components, rather than any single component, is most important in preventing proteinuria.[51]

Proximal renal tubules

Proximal renal tubular cells perform a variety of crucial physiologic functions. However, this discussion will focus primarily on their role in protein reabsorption. The glomerulus is considered critical in limiting the passage of proteins into the urine filtrate, but the renal tubules are important in reabsorbing proteins that pass through the

glomerular filtration barrier. Most of the normally filtered proteins are reabsorbed by the cells of the proximal convoluted tubules, predominantly in the S1 and S2 segments, via receptor mediated endocytosis.[29] Currently, the identified receptors include megalin, cubilin, and the recently identified accessory protein, amnionless.[57] Megalin is in the LDL family of receptors. It contains an N-terminal domain that is involved in ligand binding, a transmembrane domain, and a cytoplasmic tail that mediates clustering in coated pits (initiating endocytosis) as well as cell signaling.[58] Cubilin lacks a transmembrane domain, and its endocytosis is thought to be mediated by both megalin and amnionless.[57] Megalin and cubilin are abundantly expressed along the proximal tubule. Interestingly, glomerular expression of these receptors has been identified in rats, although to a lesser degree than in the proximal tubules, and the expression in the glomerular cells increased with age, whereas megalin expression decreased with age in the proximal tubules.[59] The authors speculate that glomeruli may take on a larger role of albumin reabsorption in older rats.[59]

Ligands of both megalin and cubilin continue to be discovered, but at this point in time, over 50 ligands have been identified for megalin as compared to 14 ligands for cubulin.[57] Each receptor has specific ligands, but they also share many ligands. For instance, retinol binding protein, transcobalamin, neutrophil gelatinase-associated lipocalin, and α1-microglobulin are bound specifically by megalin, whereas transferrin, apolipoprotein A1, and Clara cell secretory protein are bound specifically by cubilin. Albumin, vitamin D-binding protein, and hemoglobin are bound by both receptors.[57] Affinity of the receptors for each ligand varies depending on protein charge and charge

distribution, size, and conformation, and ligands compete for binding sites on the receptors in situations of protein-overload.[27,60]

As reviewed by Marshansky, et al., after proteins bind to megalin and cubilin, the receptors cluster into clathrin-coated pits at the base of the microvilli.[60] These membrane pits are then pinched off to become endosomes, which are acidified by proton pumps and chloride channels.[60] Acidification results in dissociation of the ligands from the receptors, whereby the protein receptors are recycled back to the luminal surface of the cell, and the ligands are either degraded by lysosomal enzymes or modified and released into the blood via the basolateral surface (e.g. vitamins).[58,60] The end result of this receptor-mediated endocytosis is both protein removal from the tubular filtrate and recycling of vital proteins for re-use. As mentioned, the majority of proteins are degraded, and only minimal transport of intact protein is thought to occur, as supported by some of the first perfusion studies.[61,62] Similar to vitamins, these protein fragments and amino acids are also released into the blood on the basolateral surface.[62] Recent studies have found that the majority of the protein present in normal urine is composed of protein fragments (predominantly albumin) rather than intact protein, which is thought to be due to tubular processing as well.[63]

Proteinuria-induced renal damage

Proteinuria has been implicated in the progression of renal disease as a result of several pathogenic mechanisms. These mechanisms include: 1) misdirected filtration of the urinary filtrate secondary to podocyte loss and subsequent adhesion of the

glomerulus to Bowman's capsule, 2) obstruction of the tubular lumen due to protein casts, and 3) stimulation of inflammatory pathways due to uptake of excessive and abnormally filtered proteins by the proximal tubular cells.[64] Of these mechanisms, most emphasis has been placed on the latter pathway, with megalin and cubilin implicated as key factors. Albumin, albumin-bound molecules, and other proteins abnormally filtered in patients with glomerular disease have been shown to alter signaling mediators and transcription factors within the proximal tubular cells, which lead to release of a variety of chemokines, cytokines, and fibrogenic mediators, including endothelin-1, MCP-1, RANTES, interleukin-8, and transforming growth factor-β.[65] Signaling through megalin's cytoplasmic domain is thought to be the major trigger for many of these signaling pathways. This is supported by a study using megalin knockout mosaic mice with induced glomerular proteinuria, where cells expressing megalin showed both protein uptake and staining for several inflammatory markers while staining was absent in the cells lacking megalin.[66] In addition to altered signaling, abnormally filtered proteins may have direct toxic effects on the cell when internalized.[64] Also, reabsorption of lysosomal enzymes by the proximal tubules normally helps renew the contents of the lysosomes within these cells. Therefore, during overload proteinuria, competition for reabsorption can result in lysosomal enzyme deficiency, leading to additional protein accumulation within the cell.[67] Finally, proximal tubular cells that develop an inflammatory and fibrogenic phenotype due to the above processes are thought to undergo epithelial to mesenchymal transition, thereby contributing to interstitial fibrosis.[68]

Although it is well-accepted that proteinuria influences renal disease progression, there is substantial controversy regarding its relative contribution to interstitial fibrosis as compared with glomerular injury causing encroachment of the glomerulotubular junction (secondary to misdirected ultrafiltration).[67] For instance, one study that evaluated a transgenic megalin-deficient mouse model found that inflammatory mediators were increased in megalin-positive cells as compared with megalin-deficient cells. However, tubular degeneration and interstitial inflammation was only associated with nephrons that had a blocked or partially blocked glomerulotubular junction.[69] This finding emphasizes the importance of an intact glomerulotubular junction on the remaining nephron.

Qualitative assessment of proteinuria in human and veterinary medicine

The magnitude of proteinuria generally reflects the severity and extent of glomerular lesions, and while proteinuria is associated with a more rapid course to uremic crises or death, it does not provide a good indicator regarding the degree of tubulointerstitial damage. In addition, as mentioned previously, serum tests for renal function, such as serum creatinine, require substantial loss of normally functioning nephrons before increases will become clinically evident. Therefore, the evaluation of urinary protein patterns and specific urinary proteins (qualitative assessment of proteinuria) has attracted substantial interest in recent years as a promising tool that can provide a non-invasive assessment of tubular function and that may predict progression of CKD better than total proteinuria.[29]

Patterns of proteinuria

The pattern of proteins as visualized by urine electrophoresis can be used to help determine whether glomerular and/or tubular damage is contributing to the proteinuria. More recently, proteomic techniques, including the use of electrophoretic, chromatographic, and mass spectrometric based methods, have been used to discover novel biomarkers for glomerular and/or tubular damage in urine.[70] Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most commonly used gel-based methods to assess glomerular versus tubular patterns of proteinuria. With this technique, tubular damage in the face of normal glomerular function will reveal a pattern of proteinuria consisting of predominantly low molecular weight (LMW) proteins (<40 kDa). Glomerular damage occurring without concurrent tubular damage or dysfunction will reveal a pattern consisting of intermediate (IMW) and high molecular weight (HMW) proteins (>60-70 kDa). This glomerular pattern can be either selective, with passage of only IMW proteins (predominantly albumin) or non-selective, with passage of HMW proteins. More typically, there is damage to both the glomerular and tubular components resulting in a mixed pattern of proteinuria.[71] In both dogs and people, electrophoretic patterns have been associated with prognosis and/or the degree of tubulointerstitial damage as assessed by renal biopsy.[71-74] However, while sensitivity of the urine protein pattern for detecting glomerular and tubular damage was good, specificity was found to be relatively low in dogs.[72]

Specific urinary proteins in human medicine

In addition to protein patterns, great progress has been made in the evaluation of specific proteins to determine the localization and severity of renal damage. Certain proteins have also been used to detect decreased renal function and tubulointerstitial damage earlier than when using more conventional methods. For instance, a number of studies in people have used the selectivity index to assess the degree of glomerular damage.[29] This index uses a ratio of a HMW proteins, such as immunoglobulin G (IgG), and an IMW protein, such as albumin or transferrin. A higher ratio indicates that large proteins are able to pass through the glomerular filtration barrier, and it is associated with lack of remission in certain glomerular diseases as well as a worse prognosis, even when the magnitude of proteinuria provides no predictive information.[29] This index may also correlate with the severity of tubulointerstitial damage.[29]

While HMW proteins are thought to provide a sense of the degree of glomerular injury, LMW proteins have been used to assess the degree of tubular function and therefore tubulointerstitial damage. These proteins freely pass through the glomerulus but appear in the urine due to decreased tubular reabsorption. Some of the most studied LMW proteins in the human literature include β2-microglobulin (B2M), α1-microglobulin (α1M), and retinol binding protein (RBP). A variety of studies have shown these urinary proteins to predict the clinical course of disease better than the magnitude of proteinuria. However, B2M is only stable when urine pH > 5.5.[75] Therefore, RBP and α1M have been used as more stable alternatives.[76]

B2M is a 12 kDa protein that is a component of MHC class I molecules, whereas α1M is a 27-31 kDa protein with anti-inflammatory properties.[77] Both proteins have been studied most extensively in cases of idiopathic membranous nephropathy, where they serve as independent predictors of progression to renal insufficiency and may therefore help determine which patients should be treated with immunosuppressive therapy.[78-80] However, although urinary excretion decreased with treatment, urinary B2M and α1M post-treatment did not have any prognostic value.[81] In another study of patients with early glomerular disease due to a variety of causes, B2M was the best predictor of impaired GFR as compared with other tubular indices including urinary lysozyme, the brush border enzyme dipeptydyl aminopeptidase, and fractional sodium excretion.[82]

RBP is a 21 kDa protein that transports retinol to peripheral tissues. It circulates in the blood both free and bound to transthyretin, a 55 kDa protein. In people, it is estimated that approximately 10-15% of circulating RBP is in the free form, and therefore easily filterable, although this percentage is higher in dogs.[83] Similar to B2M and α1M, urinary RBP (uRBP) has shown prognostic significance when evaluated in patients with CKD. In patients with a variety of glomerulonephropathies who were serially evaluated over months to years, uRBP and serum creatinine were the only baseline findings that served as independent predictors of renal function loss.[84] In this study, uRBP was better than serum creatinine at predicting progression when a 6-month follow-up evaluation was performed. Furthermore, uRBP was also useful in cases where serum creatinine was initially normal.[84] However, it is interesting to note that normal

uRBP concentrations were found in the majority of patients with minimal change disease and mesangial proliferative glomerulonephritis.[84] This suggests that the appearance of uRBP may not be greatly influenced by competition for reabsorption or increased glomerular filtration of RBP bound to transthyretin in glomerular disease. Another study found uRBP to closely correlate with serum creatinine and to predict progression to renal failure.[85] In addition, the percentage of renal tubular atrophy and fibrosis was higher in those patients with increased uRBP. However, unlike for uRBP, a statistical correlation between tubulointerstitial injury and creatinine clearance as well as disease progression could not be identified.[85] This supports the idea that urinary proteins provide a more global assessment of renal injury than renal biopsies and therefore may be more appropriate for estimating renal function.

It should be pointed out that many of the low molecular weight proteins have also been evaluated in the serum of patients with CKD, and similar to creatinine, their serum levels increase with declining renal function. Some studies have assessed the use of B2M, for instance, as a surrogate marker for GFR in children,[86] and patients with renal failure have an increase in unbound RBP with no change in the bound fraction.[87] It is possible that some of these proteins, such as RBP, contribute to the uremic metabolic syndrome that occurs with end-stage renal disease.[88] However, factors other than decreased urinary filtration may increase the serum concentration of these proteins. For example, serum B2M may be increased due to non-renal neoplastic and inflammatory diseases, whereas RBP may be increased with obesity.[87,89]

Other "tubular" proteins that have been shown to be useful in the evaluation of renal disease are those that are produced and released by the tubular cells in response to injury. These proteins include brush border, lysosomal, and cytosolic enzymes that are released secondary to tubular damage (e.g., N-acetyl-β-D-glucosaminidase (NAG), γ-glutamyltransferase (GGT), alkaline phosphatase (ALP), and glutathione-S-transferase (GST)), in addition to proteins that are upregulated with tubular injury (e.g., kidney injury molecule-1 (Kim-1) and neutrophil gelatinase-associated lipocalin (NGAL)). Tubular enzymes have been studied extensively over the years, and urinary NAG in particular has been found to be more sensitive for renal dysfunction than serum creatinine, total protein excretion, or several other urinary enzymes in patients with chronic glomerulonephritis and pyelonephritis.[90] Urinary NAG was also found to predict renal disease progression and response to therapy in patients with several different glomerular diseases.[91]

While renal tubular enzymes have shown promise in the evaluation of tubular damage, there is debate as to whether enzymes that also circulate in the blood should be evaluated in patients with glomerular disease. Large urinary enzymes are considered to be solely of renal origin when the glomerular filtration barrier is intact. However, in cases of non-selective proteinuria, plasma contribution to the urinary enzyme activity is possible. This dilemma has been evaluated in several studies for NAG (150 kDa), where isoenzyme analysis can help determine renal versus plasma origin of the enzyme activity in urine, since isoenzyme A2 predominates in kidney tissue whereas isoenzymes A1 and A2 are present in approximately equal proportions in the plasma.[92] Uniformly, in

patients with proteinuric renal disease, urine isoenzyme analysis was more similar to that found in renal tissue than in the plasma, with the isoenzyme A2 predominating.[92-94] These studies support the use of urinary NAG for evaluating tubular function and damage in patients with proteinuric disease. However, it is important to note that the increase in NAG may be due to increased lysosomal activity secondary to increased protein uptake instead of, or in addition to, tubular damage.[95]

Kim-1 and NGAL represent two recently identified markers of tubular injury, and both of these proteins have been more extensively evaluated in patients with acute kidney injury, with relatively few studies in patients with chronic kidney disease. KIM-1 is a recently described membrane protein whose expression is upregulated with tubular damage,[96] and it has shown promise as a marker of tubular injury in proteinuric renal disease.[97,98] NGAL is a 25 kDa protein that most notably binds siderophores and is upregulated within several types of damaged epithelial cells, including proximal renal tubular cells.[99] However, it also circulates in the plasma, and while urinary NGAL has shown promise in the evaluation of patients with CKD, it is highly influenced by proteinuria alone.[99] Therefore, its increase in proteinuric disease is likely due to a combination of both decreased tubular absorption as well as increased tissue expression.

Since progression of renal disease assumes continual nephron damage and loss, these markers of direct tubular injury may provide information regarding the degree of injury that is occurring at any one time. This information is different than the functional information provided by the low molecular weight proteins. It follows, however, that the direct tubular injury markers might markedly fluctuate depending on the conditions at

the time of measurement. In addition, as fewer nephrons are present to produce the various enzymes and proteins, their urinary excretion may also decrease.[100] Therefore, while their measurement provides useful and unique information, it would not be surprising for these proteins to show a lack of correlation with disease progression.

Specific urinary proteins in veterinary medicine

While numerous studies have evaluated specific urinary proteins in people, far fewer studies have been performed about such proteins in dogs. In these studies, an increase in a number of urinary proteins has been demonstrated in dogs with CKD, including NAG, GGT, RBP, B2M, α1M, lysozyme, vitamin D-binding protein, transthyretin, IgG, transferrin, and glycyl-prolyl dipeptidyl aminopeptidase.[101-107] Additionally, the amount of Tamm-Horsfall protein, which is normally produced by the distal tubule, has been shown to be decreased in the urine of dogs with CKD.[107]

Of the above proteins, the most studied in veterinary medicine is NAG. NAG is a lysosomal enzyme present in proximal renal tubular cells that is released from the cells secondary to tubular damage. An increased urinary NAG:creatinine ratio has been reported in dogs with CKD as well as in dogs with pyometra and acute tubular damage due to renotoxic agents.[103,105,108,109] Increases have also been found in cats with CKD and hyperthyroidism.[110-113] However, NAG was not a unique significant factor in predicting the development of azotemia in geriatric cats, supporting a lack of prognostic value in this population.[113]

RBP has also been evaluated in several studies in both dogs and cats. Thus far, all of the studies that have evaluated RBP in dogs have found the urinary RBP:creatinine

ratio to be increased in dogs with CKD as compared with healthy dogs.[103,107,114] In one of these studies, RBP strongly correlated with azotemia.[103] Increases in urinary RBP were also found in cats with CKD and hyperthyroidism, and similar to dogs, there was good correlation with the degree of azotemia present in these patients.[115-117]

While measurement of urinary proteins in veterinary medicine appears promising, the number of studies in dogs with renal disease is limited, and most of these studies thus far have been limited to case reports and cross-sectional studies. Currently, the only serial evaluation of specific urinary proteins in dogs involved the semi-quantitative analysis of RBP, B2M, vitamin D-binding protein, α1-microglobulin, albumin, transferrin, apolipoprotein A1, and IgG in dogs with X-linked hereditary nephropathy.[104] This study reported a progressive increase in all proteins except albumin and IgG.[104] However, serial quantitative analysis of urinary proteins and correlation of these proteins with standard measures of renal function have not been performed. Therefore, further evaluation is needed to better establish the role of urinary proteins in the early diagnosis and monitoring of renal disease.

Canine X-linked hereditary nephropathy

For this dissertation, a canine model of proteinuric renal disease caused by X-linked hereditary nephropathy (XLHN) was used. XLHN was identified in a family of mixed-breed dogs from Navasota, TX.[44] The disease in this family is caused by a ten base pair deletion within the coding region of the COL4A5 gene located on the X chromosome.[45] This gene encodes the α5 chain of type IV collagen, and the deletion

results in a premature stop codon and subsequently altered transcription of this gene.[45] Affected dogs therefore lack the normal α3-α4-α5 type IV collagen chain network within the glomerular basement membrane, and instead have an abundance of the α1-α1-α2 network of type IV collagen.[44]

Electron microscopy imaging of the glomerulus in affected dogs reveals diffuse thickening and splitting of the basement membrane, which is characteristic of this particular disease, as well as podocyte foot-process fusion.[44] These changes result in altered glomerular permselectivity, which is characterized by the development of early and marked proteinuria.[44] Hemizygous (affected) males exhibit rapidly progressive disease that causes them to develop end-stage renal disease (ESRD) at approximately 1 year of age. Affected males develop persistent glomerular proteinuria between 3 and 5 months of age, and the magnitude of proteinuria progressively increases until relatively late in the disease process. This proteinuria initially demonstrates a glomerular pattern; however, progressive tubular damage contributes to the mixed glomerular/tubular pattern observed later in the disease process. Their progression to ESRD is observed histologically by progressive tubulointerstitial inflammation and fibrosis in addition to glomerular sclerosis and tubular degeneration, atrophy, and necrosis.[44] In contrast, most heterozygous (carrier) females live a normal lifespan, but some may progress to ESRD. As young adults, the carrier females typically remain healthy with good renal function as indicated by a normal serum creatinine concentration (0.9-1.1 mg/dl) and urine specific gravity > 1.035. However, they do develop proteinuria early in life, and the magnitude of proteinuria remains stable during early adulthood (1-5 years of age).

This family of dogs provides an opportune model for studies involving naturally occurring glomerular disease. The affected males, for example, can be used to study progressive CKD due to glomerular disease. Although their structural defect is a specific lesion within the GBM, the glomerular and tubulointerstitial lesions that develop are comparable to lesions occurring in dogs with progressive renal disease regardless of the cause. The carrier females, on the other hand, are useful for studying non-progressive proteinuric nephropathy. Evaluation of the day-to-day variability of urine proteins, for example, is possible given their stable magnitude of proteinuria over long periods of time. In addition, those females that do not demonstrate clinical evidence of disease progression may be useful for evaluating the influence of glomerular proteinuria on the presence of tubular proteins. Therefore, this canine model was instrumental in carrying out the objectives outlined below.

Specific objectives

The overall theme of this project was to identify methods that could aid veterinarians in the clinical diagnosis and monitoring of proteinuric CKD. Three specific objectives were carried out in order to accomplish this goal: 1) to determine the basal fluctuation of the UPC in dogs with an unchanging magnitude of proteinuria, 2) to search for novel urine biomarkers for early renal injury, and 3) to serially assay selected promising urine biomarkers of renal injury to evaluate their use during the early detection of and/or monitoring progression of CKD in dogs.

To determine variability of the UPC, a unique statistical model was applied to results from serial urine samples collected from carrier XLHN female dogs. Day-to-day variability in their UPC was calculated, and the number of urine samples necessary to provide a true estimate of the UPC was determined.

Novel biomarkers were identified in the urine of XLHN males by using proteomic techniques. Urine protein profiles were compared between two time points that spanned the development of early tubulointerstitial injury. Using two-dimensional gel electrophoresis and surface-enhanced laser desorption ionization, several proteins and peaks were identified that have not previously been associated with renal disease in dogs.

To accomplish the third objective, a number of proteins were selected that have shown promise in the human and veterinary literature as biomarkers of CKD. Commercially available assays were utilized when possible, and these assays were all analytically validated for use in canine urine. The proteins were then evaluated serially in the urine of male dogs affected with XLHN and their normal siblings. In the affected males, analysis spanned the pre-clinical stage until end-stage renal failure was reached. Correlations were made with standard measures of renal function, including serum creatinine, glomerular filtration rate, histologic analysis, and UPC. Based on these results, the usefulness of the selected urinary biomarkers for detection and monitoring CKD progression in dogs was evaluated.

CHAPTER II

DAY-TO-DAY VARIATION OF THE URINE PROTEIN:CREATININE RATIO IN FEMALE DOGS WITH STABLE GLOMERULAR PROTEINURIA CAUSED BY X-LINKED HEREDITARY NEPHROPATHY*

Overview

Proteinuria has been shown to be a predictor of morbidity and mortality in people, dogs, and cats with CKD. An increased magnitude of proteinuria likely serves as both an indicator of more severe renal lesions in addition to contributing to renal disease progression. Therefore, treatment of proteinuric CKD in dogs is largely aimed at reducing the magnitude of proteinuria, as estimated by the urine protein-to-creatinine ratio (UPC). However, in order to know whether a UPC measurement indicates an improvement or worsening of proteinuria as compared to a previous UPC measurement, one must have a sense of the variability of the UPC in the absence of disease progression or modifying therapies. In order to address this issue, this study describes the day-to-day variability of the UPC in heterozygous female dogs with X-linked hereditary nephropathy. These dogs have proteinuria and renal function that remains stable for prolonged periods of time, such that their day-to-day variability is not influenced by

*Reprinted with permission from Day-to-Day Variation of the Urine Protein:Creatinine Ratio in Female Dogs with Stable Glomerular Proteinuria Caused by X-Linked Hereditary Nephropathy by M. Nabity, M. Boggess, C. Kashtan, and G. Lees, 2007. Journal of Veterinary Internal Medicine, 21, 425-430, Copyright 2007 by John Wiley and Sons.

renal disease progression. The reference change value was calculated for these dogs to represent the amount of change that could be explained by biological variation, with 95% confidence. In these dogs, in order to exceed expected day-to-day variation in the UPC, the subsequent UPC value must increase or decrease by 35-50% when the UPC > 3 (range 3-12), and 50-80% when the UPC < 3 (range 0.5-3), with a higher percent change required the lower the UPC. In addition, measurement of the UPC on a single urine sample is adequate to estimate the UPC when values are ≤ 4. However, in order to adequately estimate the true UPC value, the average UPC determined from 2-3 urine samples is necessary when the UPC is 4-8, and from 4-5 urine samples when the UPC is > 8. These data currently provide the only calculated guidelines available to practitioners when monitoring patients with proteinuria.

Introduction

The UPC has become widely used in veterinary medicine as an index of magnitude of proteinuria in dogs in the 2 decades since studies first validated its use for this purpose.[17,18,118] A UPC persistently ≥ 0.5 is indicative of an abnormal degree of proteinuria, whereas a UPC < 0.5 is consistent with absence of significant proteinuria.[12] Greater magnitudes of proteinuria correlate with severity and progression of renal disease in humans and animals, including dogs.[19] Additionally, recent studies have shown that various therapeutic interventions, such as administration of angiotensin converting-enzyme inhibitors or dietary modifications, can reduce magnitude of

proteinuria and slow progression of renal disease.[21-24] Because of such findings, veterinarians have begun to recognize potential benefits of reducing proteinuria in dogs with a variety of renal diseases, and treatment of dogs to reduce the magnitude of their proteinuria has been recommended.[12] Serial UPC monitoring is used to assess treatment efficacy, disease progression, and prognosis in proteinuric dogs. Many important clinical decisions for dogs with proteinuria hinge on an ability to detect clinically important changes in serial UPC values.

Determining whether a UPC value has changed on serial measurements requires knowledge of the variability of the UPC when the magnitude of proteinuria is unchanging. To the authors' knowledge, there are no published reports of day-to-day biological variability of the UPC in proteinuric dogs (UPC $\geq$ 0.5). One reason for this is because a wide range of diseases can cause proteinuria in dogs, and the majority of these diseases have a variable effect on the progression of proteinuria. Thus, random biological variation cannot be easily distinguished from other sources of variation. In this study, we used dogs with a rare but well-defined renal disease causing a stable magnitude of glomerular proteinuria for extended periods of time in order to assess the random day-to-day fluctuation in the UPC ratio.

The goals of this study were to establish guidelines for the expected variability in the UPC of dogs with unchanging proteinuria and the number of measurements needed to obtain a reliable estimate of the actual UPC value.

Materials and methods

Dogs

Retrospective analysis was performed using data collected between January, 1999, and October, 2002, from 48 young-adult (12-32 months old) heterozygous (carrier) female dogs with X-linked hereditary nephropathy (XLHN). The dogs were members of a single family maintained in a colony at Texas A&M University since 1997. XLHN in this kindred is caused by a nonsense mutation in the *COL4A5* gene that encodes the α5 chain of type IV collagen, which is a crucial component of normal glomerular basement membranes (GBM).[45] The salient clinical and pathologic features of the nephropathy that occurs in dogs with this gene defect have been described.[44] In carrier females, these features include mosaic expression of type IV collagen peptides that are normally found in the GBM and onset of persistent glomerular proteinuria between 3 and 6 months of age.

All puppies produced in the colony were raised using a standardized protocol for feeding, husbandry, routine health care and socialization. Additionally, renal function of all carrier females was monitored by measuring serum creatinine concentration every 3 months. A few (< 10%) carrier females had intermittent or persistent increases in serum creatinine concentration before 3 years of age, but the great majority (> 90%) of the carrier females raised within the colony remained clinically healthy and maintained good renal function as adolescents and young-adults. Despite their proteinuria, these dogs had urine specific gravity values indicative of adequate urine concentrating ability (≥ 1.035) and stable serum creatinine concentrations in the middle of the reference range. Forty-

eight of the young adult XLHN carrier females having these attributes were selected for the studies of proteinuria that generated the data retrospectively analyzed for this report.

During studies, dogs were housed in runs in a temperature-controlled room with a 12-hour light-dark cycle, and they were fed once daily in the afternoon. Dogs were leash walked outside or were permitted short periods of unrestricted access to an exercise area daily.

Data collection

Several studies were conducted in a similar fashion to examine the influence of various factors on magnitude of proteinuria in dogs with glomerular disease. The study protocols were reviewed and approved by the Texas A&M University Laboratory Animal Care Committee, and results of the studies have been published[119] or reported at scientific meetings.[120-122] Magnitude of proteinuria was assessed in each dog by measuring the UPC once daily on each of the last 3 days of various periods during which the dog was maintained or treated in a specified manner. All dogs were evaluated after 1, 2, or 4 weeks without treatment. Additionally, some studies included treatments that were given for periods of 4 or 6 weeks during which the dogs were evaluated on the last 3 days of each successive 2-week interval. After treatment (being fed a specified diet or given prednisone), dogs were either crossed-over to a second diet or observed after cessation of prednisone administration for additional 4- or 6-week periods during which evaluation at 2-week intervals continued. Changes in the UPC typically occurred sufficiently quickly after changes in treatments so that mean values had stabilized by 2 weeks after initiation or cessation of treatment. An aliquot of urine obtained by

cystocentesis was submitted for quantitative aerobic bacterial culture to verify absence of urinary tract infection in each dog at the outset of her study protocol and again at the end of her protocol if she was studied for > 1 week.

The combined data available from the 48 dogs used in these studies included a total of 183 3-day evaluation periods and 549 UPC determinations. All 48 dogs were evaluated 1-2 times before treatment, resulting in 75 3-day evaluation periods (225 UPC determinations) without treatment.[119-122] Twelve dogs were treated with a high or low protein diet, resulting in 84 3-day evaluation periods (252 UPC determinations) during the 14-week treatment period.[119] Six dogs were treated with prednisone (2.2 mg/kg PO q24h), resulting in 24 3-day evaluation periods (72 UPC determinations) during the 8-week treatment and post–treatment periods.[122]

Sample collection and assay

All urine samples were collected by cystocentesis during the morning; *i.e.*, before the dogs were fed each day. Samples were refrigerated immediately after collection and assayed within 6 hours. Urine was centrifuged (300 x g for 5 min), and urine protein and creatinine concentrations in the supernatant were measured using a dry-film chemistry auto-analyzer (Vitros 250, Johnson & Johnson Co., Rochester, NY). Urine protein (mg/dL) was determined by a colorimetric method using a pyrocatechol violet-molybdate complex. Urine creatinine (mg/dL) was determined by a colorimetric enzymatic method using creatinine amidohydrolase. Samples were diluted by the instrument according to manufacturer's specifications when necessary.

Analytical variability

Analytical variability of UPC was determined using a fresh urine sample obtained from three XLHN carrier female dogs, one each having a low (0.5), moderate (2.7), or high (7.9) UPC. Each of these samples was measured six times over 72 hours (once in the morning and once in the afternoon of each day) to assess inter-assay variability. Each sample was also measured 10 consecutive times during a single instrument run to determine intra-assay variability. Samples were stored at 4^{o}C between analyses. Assays were performed for only 3 days because urine protein determinations may be unreliable after more than 3 days of sample storage at 4^{o}C according to the instrument guidelines, and freezing may precipitate proteins.[123] Analytical variability was also assessed using control standards that were measured each day as UPC measurements were performed on clinical patients over the course of one month. The creatinine concentrations of the control standards were comparable to those of the urine samples; however, the protein concentrations of the control standards were much lower than those of the moderate- and high-UPC urine samples, resulting in much lower overall UPC values for the control standards than for the canine urine samples used to assess analytical variability.

The variance components for analytical variability were estimated using one-way random effects ANOVA. Using the representative low, moderate and high UPC urine samples, the estimated intra-assay standard deviation (s^{w}) = 0.07 and intraclass correlation coefficient (ICC) = 0.99965 (0.99895, 1.00036), and inter-assay s^{w} = 0.06 and ICC = 0.99973 (0.99915, 1.0003). Using the control standards, the estimated inter-

assay s^w = 0.03, and the ICC = 0.99374 (0.97611, 1.01136). Compared to biological variability, analytical variability is optimally low,[124] and we assume that its contribution to total variation is minimal. Thus the analytical variability was not incorporated separately into the model but was incorporated into the between and within evaluation variance components described below.

Statistical methods

The two major sources of variation are biological variation and analytical variation. Biological variation includes that due to intra-individual and inter-individual variation (within and between evaluation variance, respectively). The within evaluation variance component (σ_w^2) reflects the variability due to repeated measurements on the same dog within a given evaluation period (3 consecutive daily measurements). σ_w was modeled as a function of mean UPC using $\sigma_w = \sigma \times UPC^{\theta}$, also known as a power of the mean model.[125] This model was chosen because the standard deviation (σ_w) of the UPC measured over 3 days increases with higher UPC values in all dogs, whether untreated or treated, and is therefore not constant with respect to the UPC level (Figure 2). If the variability of the UPC had been constant over the full range of values, then it could have been readily estimated with a straightforward technique (*e.g.*, random effects ANOVA). However, variation that increases in proportion to the mean is a common phenomenon with values whose lower limit is bounded by zero, which is the case with serum and urine chemistry analytes. This requires the use of other methods to estimate variance.[125-127]

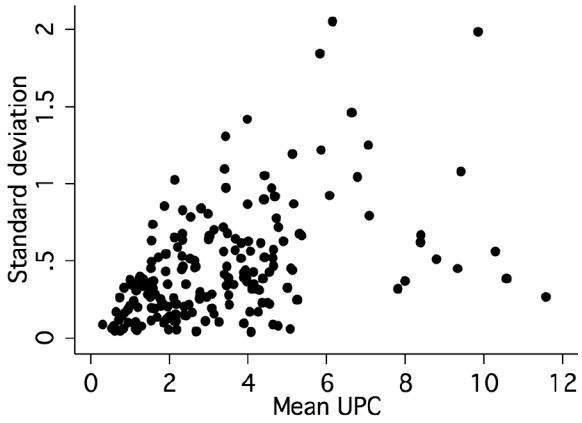

Figure 2. Scatter diagram of the standard deviations of urine protein:creatinine ratio (UPC) values within 3-day evaluation periods, plotted against the mean UPC value. Standard deviation increases significantly as the mean UPC increases.

The between evaluation variance component (σ_b^2) reflects the variability due to measurements obtained from different evaluation periods, as a result of measurements obtained over a period of weeks to months, either from the same dog or from different dogs. This component was modeled with a random effect assuming a normal distribution for the 3-day evaluation period. Since the sample contained multiple evaluations performed on the same dog, bootstrapped standard errors were used to take into account the probable correlation.[128] All estimations were performed with Stata 9® (Intercooled Stata 9.0, Stata Corporation, College Station, TX). The estimate of the standard deviation (σ_w) is denoted as s_w; similarly, the estimate of σ_b is denoted as s_b.

The estimate of the standard deviation, s_w, was used to calculate the reference change value (RCV): $RCV = 1.96 \times (2)^{1/2} \times (s_w^2)^{1/2} = 2.77 \times s_w$. The RCV represents the amount of change that is reasonably explained by biological variation. Therefore, if no change in the magnitude of proteinuria has occurred, serial measurements should fall

within the interval created by adding or subtracting the RCV from the original UPC, with 95% confidence.[127,129] The reference change value percent (RCV%) was also determined: RCV% = RCV/UPC. This value is the percent change in the UPC necessary to determine an increase or decrease in UPC.

The RCV and RCV% are meant for comparison of two single UPC measurements. However, the RCV can be extrapolated to include multiple averaged UPC measurements. If the UPC from several urine samples have been averaged in order to obtain a more accurate estimation of the UPC, then the RCV can be calculated by: $2.77 \times (s_w / \sqrt{n})$, where n is the number of averaged measurements and s_w is their standard deviation.

The intraclass correlation coefficient (ICC) = $\sigma_b^2/[\sigma_b^2 + \sigma_w^2]$. This reflects the reliability of repeated measurements, so that a higher ICC is suggestive of a more reliable measurement.[127] The Spearman-Brown prediction formula relates the ICC to the number of measurements (k) required to achieve a given reliability:[130] $ICC^1 = k \times ICC/[1 + ((k-1) \times ICC)]$. Acceptable reliability was set at $ICC^1 = 0.9$. The standard error for the quantities described above were calculated by the delta method.[131]

Because UPC measurements were obtained from some dogs that were given treatments (prednisone and varying diets), the effect of treatment on variance of UPC was analyzed. Treatment effect on variance was determined by comparing the s_w of the untreated evaluations to the s_w of each treatment group. The 95% confidence intervals for the s_w of each treated group largely overlapped with those for the untreated group

supporting the absence of treatment effect on the variability of the UPC (not shown). Therefore, all observations were pooled over treatments for the analysis.

Results

The standard deviation estimates for UPC day-to-day variability were s_w: 0.24 x $UPC^{0.74}$ and s_b: 2.01 (1.78, 2.23). Normality of the residuals was verified by the use of a normal probability plot.

Applying the s_w results, RCV = 2.77 x (0.24 x $UPC^{0.74}$) and RCV% = [2.77 x (0.24 x $UPC^{0.74}$)]/UPC. The RCV and RCV% were then plotted against the UPC value to provide continuous plots that can be used to identify significant changes from an initial UPC value between 0.5 and 12 (Figures 3 & 4). For example, to be 95% confident that the magnitude of proteinuria has increased from a baseline UPC of 5, then using the graphs, a subsequent UPC value must increase by at least 48% or 2.4, resulting in a value of 7.4. By adding and subtracting the RCV from the initial UPC, one can determine the critical values that indicate the largest possible deviation from baseline that can be reasonably expected due to random biological variation. Subsequent measurements above and below these critical values are far enough from baseline to support a significant change in the UPC (Table 1). To summarize, at low UPC values (near 0.5), a minimum change in the UPC of up to 80% is required to demonstrate a significant difference ($p < .05$) in serial values whereas at high UPC values (near 12), a minimum change of 35% is necessary.

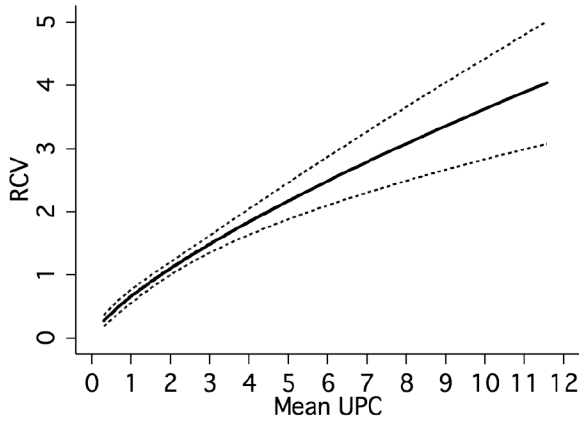

Figure 3. Graph of the absolute reference change value (RCV) for urine protein:creatinine ratio (UPC) plotted against the initial UPC value. The RCV represents the degree of change necessary to exceed day-to-day UPC variability, and it increases as the UPC increases. Solid line, RCV; dotted lines, 95% confidence interval.

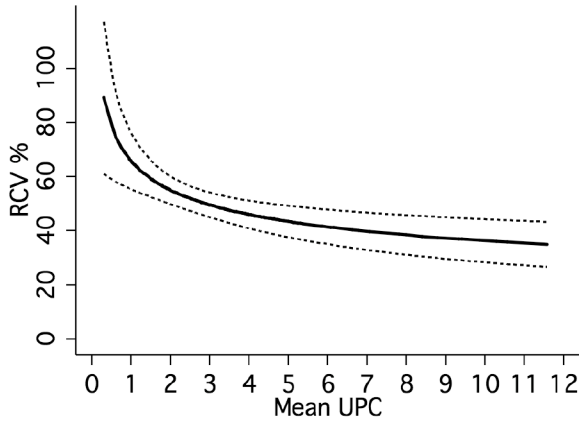

Figure 4. Graph of the reference change value expressed as a percentage of the UPC (RCV%). RCV% decreases as the UPC increases. Solid line, RCV%; dotted lines, 95% confidence interval.

Table 1. Subsequent UPC values required to demonstrate a significant (95% confidence) decrease or increase in UPC following an initial determination.

	Subsequent UPC	
Initial UPC	Value required to demonstrate significant decrease	Value required to demonstrate significant increase
0.5	<0.1	>0.9
1	<0.3	>1.7
2	<0.9	>3.1
3	<1.5	>4.5
4	<2.1	>5.9
5	<2.8	>7.2
6	<3.5	>8.8
7	<4.2	>9.8
8	<4.9	>11.1
9	<5.6	>12.4
10	<6.3	>13.7
11	<7.1	>14.9
12	<7.8	>16.2

The ICC and the Spearman-Brown prediction formula were used to estimate the number of averaged UPC determinations needed to obtain a reliable estimate of the UPC. Results revealed that separate urine samples give very similar results at low UPC values; however, results from separate urine samples vary more widely at larger UPC values. Table 2 lists the number of UPC determinations needed to obtain an estimated ICC $\geq$ 0.9 for various UPC measurements. In general, one measurement is adequate to estimate the UPC when values are $\leq$4. An average of 2-3 UPC determinations is necessary when the UPC is 4-8, and 4-5 averaged measurements are necessary when the UPC is > 8 to adequately estimate the true UPC value.

Table 2. Estimated number of urine samples necessary to obtain a reliable estimate of the true UPC value (ICC ≥ 0.9).

UPC Value	Number of urine samples*	Estimated ICC	95% Confidence interval
0.5	1	0.99	(0.99, 1.00)
1	1	0.99	(0.98, 0.99)
2	1	0.96	(0.94, 0.99
3	1	0.93	(0.89, 0.98)
4	1	0.90	(0.84, 0.96)
5	2	0.93	(0.88, 0.98)
6	2	0.91	(0.84, 0.97)
7	3	0.92	(0.86, 0.98)
8	3	0.91	(0.84, 0.98)
9	4	0.92	(0.86, 0.98)
10	4	0.91	(0.83, 0.98)
11	4	0.90	(0.81, 0.98)
12	5	0.90	(0.83, 0.98)

ICC, intraclass correlation coefficient
*When the number of samples is ≥2, the UPC values for all samples are to be averaged to obtain the best estimate of the true UPC value.

Discussion

Most of the carrier female dogs with XLHN raised within the colony remained clinically healthy and maintained good renal function as young adults (1-5 years of age). In addition, their prevailing magnitude of proteinuria as estimated by determining their UPC value averaged over a 3-day period (in order to minimize the confounding effects of random day-to-day variation) remained stable from week-to-week and month-to-month. During such long periods of time with little or no evidence of any substantial change in renal disease severity, most or all of any short-term (day-to-day, week-to-week, or month-to-month) change in UPC value could be attributed to random biological variation or to effects of treatments on the prevailing magnitude of proteinuria rather

than to a fundamental change in the severity of the underlying primary disease. The validity of this assumption was supported by the results of studies that used cross-over experimental designs to investigate the influence of various treatments on the magnitude of proteinuria in these dogs. When dogs in such experiments returned to treatments or conditions under which they initially had been evaluated weeks or months previously, their proteinuria consistently returned to the magnitude that had been observed before.[119] That rationale also underlies our use of data from those studies to assess biological variation of the UPC in this report. Because of the slowly progressive nature of the disease in these dogs and the lack of evident treatment effect on variance, most or all of the day-to-day variation in the UPC observed during 3-day evaluation periods was reasonably attributed to random biological variation rather than to fundamental changes in magnitude of proteinuria due to altered disease status or treatment effect.

The development of the appropriate statistical model to use in this study was challenging. Coefficient of variation (CV) has historically been used in the medical field to express the variability of an analyte, and CV was used in most of the studies that address UPC variability in humans.[132,133] However, this method does not allow for the separation of total variance into components. Separation into variance components is necessary because it allows a statistical model to be developed that predicts the expected variation of the UPC over a range of values. In addition, a model that could account for unequal variance was required. In the present study, maximum likelihood combined with the power-of-mean model allowed for calculation of the variance components while addressing the unequal variation that exists along the range of UPC values.

Dogs with chronic kidney disease that are azotemic and have a UPC > 0.5 or nonazotemic dogs with a UPC > 2 should be treated with diet, omega-3 fatty acids, and renoprotective agents, and dosing strategy may be partially determined by the response of the UPC to treatment.[12] Therefore, it is important to determine whether changing serial UPC measurements are clinically important. The reference change value (RCV) in human medicine is becoming widely used to determining biological variance of biochemical analytes.[134,135] RCV is defined as the statistically significant difference between two consecutive test results in an individual patient, and it is helpful to determine if increased or decreased serial values are likely to represent true changes or if they are compatible with biological variation.[129] In this study, the RCV was used to determine critical values for which measurements above and below represent statistically significant changes from the baseline UPC (Table 1), and the results provide the first quantitative guideline for assessing serial UPC values in proteinuric dogs.

Based on this study, the RCV graphs can be used to determine if a subsequent measurement is likely increased or decreased in XLHN dogs with a baseline UPC between 0.5 and 12. With the RCV graphs, an absolute change can be obtained from Figure 3 whereas a percent change can be obtained from Figure 4, depending on the preference of the clinician and perceived ease of use. Variability of the UPC in proteinuric dogs outside the range represented in the graphs still needs to be determined.

When a serial UPC value is outside the calculated RCV based on the initial UPC measurement, it is highly likely that a true change in the UPC has occurred. However, if the UPC value is within the limits set by the RCV, serial monitoring becomes important

to distinguish random fluctuation from a true, but small increase or decrease in the magnitude of proteinuria. For example, if a clinician finds that the UPC value in a patient has increased from 5 to 6, there is no evidence based on our data to support that this increase is significant. However, if the UPC increases from 5 to 6, and then to 7, the increasing trend is worrisome despite each serial value falling within the acceptable range for random day-to-day variation as compared to the initial UPC value of 5. In this case, a change in treatment or further evaluation to determine the cause for the increase may be warranted.

The dogs in this study were genetically related, and during the 3-day evaluation periods they were subjected to similar exogenous influences and preanalytical factors that can influence the UPC. It is this highly controlled environment that makes this study relatively accurate in its estimation of the day-to-day variability in dogs with XLHN. However, the UPCs of a random sample of dogs with a variety of naturally occurring disease processes and cared for by different owners may demonstrate more variability in their UPC due to these extra factors incorporated into the estimated day-to-day variability. In addition, dogs with other glomerulopathies may demonstrate different random biological day-to-day UPC variation than dogs with XLHN. Therefore, the variability of the UPC in dogs with other glomerular diseases still warrants investigation in order to determine to what extent these guidelines are applicable to other disease states.

The second purpose of this study was to determine the number of urine samples that should be averaged to obtain an accurate estimate of the UPC. Gibb and colleagues

recommended averaging 5 urine samples to decrease UPC variation in humans, but they did not address whether a different number of samples is recommended for low and high UPC values.[136] Based on the results of our study, when the UPC is < 4, reliability of that value is high, and therefore, the UPC needs to be measured only once to obtain a reliable value (Table 2). This finding suggests that a dog can generally be classified as normal or proteinuric at one point in time based on a single sample, with the caveat that additional measurements may be needed to confirm persistence of proteinuria. Also, serial monitoring is recommended to confirm mild proteinuria if the UPC value is near 0.5. At higher UPC values (>8), we find similar results to Gibb et al, where 4-5 measurements may need to be averaged to obtain a reliable UPC value. However, in many cases it may be impractical to take 5 separate urine samples to obtain a single UPC estimate due to cost, time, and/or a rapidly progressing disease process. Even averaging 2 measurements greatly increases the accuracy of the UPC estimate, and when possible, we recommend averaging measurements obtained from 2-3 separate urine collections or pooling 2-3 urine samples (within a 3-day period) when the UPC is > 4.

Guidelines are currently not available for timing of sample collection with repeated UPC measurements. Many studies have found good correlation of the UPC with the total 24-hr protein excretion in urine samples collected at random;[18,137] however, UPC variability was not evaluated. One study in humans found that the UPC had the lowest day-to-day variation in early morning urine samples compared to bedtime samples.[133] No such studies have been performed in animals. Therefore, when multiple samples are being collected, whether to assess the true UPC value or for long-term

monitoring, we recommend taking steps to minimize factors that can influence UPC variability. These steps include sampling at the same time of day and before eating or strenuous exercise, using fresh samples collected by the same technique each time, and analyzing the UPC using the same laboratory and the same instrument for all evaluations from a single patient.

The results of this study may provide practical recommendations for practitioners to use when monitoring UPC values, although variability of the UPC in dogs with other glomerular diseases has yet to be determined. When values are not deemed significantly different based these recommendations, increasing or decreasing trends in the UPC remain important to determine disease progression and/or response to therapy. In addition, the UPC should be used in conjunction with patient assessment via physical examination and other clinical parameters in order to best determine treatment options.

CHAPTER III

PROTEOMIC ANALYSIS OF URINE FROM MALE DOGS DURING EARLY STAGES OF TUBULOINTERSTITIAL INJURY IN A CANINE MODEL OF PROGRESSIVE GLOMERULAR DISEASE

Overview

The urine proteome is defined as the total composition of proteins and peptides within the urine, and it can be explored by using proteomic techniques. The use of proteomic techniques for the identification of tissue biomarkers is only a relatively recent area of research, spurred on by the advent of highly sensitive mass spectrometry. An important concept is that these techniques are excellent at providing numerous data about the protein content of a tissue without the need for prior assertions or cross-reacting antibodies. However, this information should be verified using more specific assays. Therefore, these tools have been utilized to both identify patterns of disease as well as to identify specific proteins that can then be verified by separate assays. A variety of tools are available for proteomic analysis, and this study describes the analysis of urine proteins in male dogs with XLHN using 2-dimensional gel electrophoresis and surface enhanced laser desorption ionization (SELDI). The samples chosen for analysis were obtained from two early time points in their disease progression. The dogs were markedly proteinuric at both time points supporting extensive glomerular injury, but azotemia was evident only at the latter time point. Therefore, these samples provided an opportunity to target early biomarkers of tubulointerstitial injury. Both techniques

identified known biomarkers of renal disease, including retinol binding protein. However, the 2-dimensional analysis identified several proteins that have not previously been implicated in renal disease in dogs and that have only been minimally studied in renal disease in people, including hemopexin, fetuin A, and gelsolin. In addition, some of the proteins identified have not previously been implicated in renal disease at this point in time. SELDI identified a number of promising peaks that warrant further investigation. These findings may help direct future studies of renal disease in dogs.

Introduction

Chronic kidney disease (CKD) is a major cause of morbidity and mortality in dogs, and it is thought that dogs with advanced disease represent only a fraction of all dogs with CKD.[1,2] It is also recognized that the degree of histologically detectable tubulointerstitial damage most closely correlates with renal function.[5] However, current non-invasive methods to detect and monitor early tubulointerstitial disease are limited. When monitoring renal disease, clinicians rely primarily on serum creatinine and urea nitrogen concentrations, urine specific gravity, and the urine protein:creatinine ratio (UPC). However, azotemia and impaired urine concentrating ability are generally thought to be present only after approximately 65-75% of the renal parenchyma has been damaged.[9] An increased UPC can occur due to either glomerular or tubular injury, and it is most often secondary to glomerular injury, particularly when the UPC > 2.[12] In recent years, several promising candidate renal biomarkers for tubulointerstitial injury have been evaluated in human medicine, and some, including retinol binding protein and N-

acetyl-ß-D-glucosaminidase, have been studied recently in dogs with chronic renal disease.[102,103,105,107,114] While results are encouraging, further investigation will be required to establish the clinical utility of these tests.

Since there currently are no sensitive and specific markers for the detection and monitoring the progression of early tubulointerstitial injury, the search for novel renal biomarkers continues. Proteomic techniques have become widely used as a powerful approach for the discovery of novel biomarkers in a variety of diseases and tissues. Proteomic analysis allows for the simultaneous detection of a wide variety of proteins, and results are not restricted to known biomarkers. In addition, proteomic analysis does not require antibodies, which are often a limiting factor in the evaluation of proteins from veterinary patients. Thus, proteomic techniques are well suited for biomarker discovery.

In veterinary medicine, few studies have been reported that used proteomic techniques to identify biomarkers for renal disease.[107,138] In these studies, the dogs evaluated were clinical patients with spontaneous renal diseases that were in different stages of disease. Also, these patients had renal diseases that were secondary to a variety of different causes. In contrast, the objective of this study was to explore the urine proteome in dogs with early progressive renal disease due to a single cause in order to identify promising biomarkers for early tubulointerstitial injury. The dogs used in this study all have a specific mutation in the gene encoding for the type IV collagen in their glomerular basement membrane. Due to this mutation, males with this defect develop renal disease that progresses to end-stage renal disease (ESRD) during adolescence, and

the clinical and histologic progression to ESRD in these dogs is similar to that in dogs with other progressive glomerular diseases. They therefore provide a unique opportunity to search for potential early markers of tubulointerstitial injury in progressive CKD without the confounding factors present in a diverse, client-owned population of dogs with a variety of diseases.

The two proteomic techniques used in this study were two-dimensional differential in-gel electrophoresis (2-D DIGE) and surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry. 2-D DIGE uses gels to separate fluorescently-labeled proteins based on charge and mass, and proteins of interest can be readily identified from spots separated on the gel using mass spectrometry. SELDI-TOF uses chromatography combined with mass spectrometry to detect proteins. It is particularly useful for detecting low molecular weight proteins and peptides, and it tends to be more sensitive than gel-based methods for detecting low-abundance proteins. However, its utility in detecting high molecular weight proteins is more limited.

The aim of the present study was to use these two proteomic techniques in the prospective evaluation of a well-defined canine model of CKD in order to identify novel urinary biomarkers of early tubulointerstitial injury in canine progressive renal disease.

Materials and methods

Animals

Six male, mixed-breed dogs with X-linked hereditary nephropathy (XLHN) were used in this study. These dogs were members of a single family maintained in a colony at Texas A&M University since 1997. XLHN in this kindred is caused by a nonsense mutation in the COL4A5 gene located on the X chromosome that encodes the ∝5 chain of type IV collagen, which is a crucial component of the normal glomerular basement membrane (GBM).[45] In affected males, the salient clinical and pathologic features of the nephropathy include absence of the normal collagen IV network in the GBM, development of marked proteinuria, and rapidly progressive renal disease that typically results in end-stage renal failure at about 1 year of age.[44]

All puppies produced in the colony were raised using a standardized protocol for feeding, husbandry, routine health care and socialization. During studies, dogs were housed in runs in a temperature-controlled room with a 12-hour light-dark cycle, and they were fed once daily in the morning after urine collection. Dogs were leash walked outside or were permitted short periods of unrestricted access to an exercise area daily. Three of the puppies were neutered at 14 wks of age, so that two dogs were neutered between the samples evaluated (as described below), and one dog was neutered before either sample collection. Three of the puppies remained intact for the duration of the study. No treatments were administered to the dogs whose samples were utilized in this study. The study protocol was reviewed and approved by the Texas A&M University Laboratory Animal Care Committee.

Urine collection

Blood and voided, mid-stream urine were collected in the morning on a weekly to biweekly basis from 6 male dogs with XLHN starting at 9 wks of age. For each time point, urine samples were collected over 3 consecutive days, and on the day of collection, each sample was centrifuged for 10 minutes at 500 x g and 4^{o}C, the supernatant was removed, and protease inhibitors were added (Complete Protease Inhibitor Cocktail, Roche Diagnostics Corp., Indianapolis, IN). Urine was frozen at -50^{o}C within 6 hrs of collection. The frozen samples were later thawed, and the samples from each 3-day collection were combined resulting in a single sample, which was then centrifuged, aliquoted, and stored at -80^{o}C. Urine protein and creatinine concentrations were measured in fresh, refrigerated pooled urine samples from the 3-day collections, and serum creatinine concentration was measured on the first day (Vitros 250, Ortho-Clinical Diagnostics Inc., Rochester, NY). Urine specific gravity was measured using a refractometer.

Urine preparation for proteomic analysis

Urine from two time points was selected for analysis based on the potential to result in detection of early biomarkers of tubulointerstitial injury: time point 1 (TP 1) was the time point after which dogs first became overtly proteinuric (UPC > 2; age range 13-25 wks) and time point 2 (TP 2) at which dogs first demonstrated mild azotemia as compared with normal male littermates (serum creatinine ≥ 1.2 mg/dl; age range 21-35 wks).

Urine samples from each time point were thawed, centrifuged for 10 minutes at 12,000 x g and 4^{o}C, and passed through a 0.45 μm syringe filter. In order to enrich for low-abundance proteins, albumin was removed from the samples by affinity chromatography (Seppro IgY HSA LC2 Column, GenWay Biotech, Inc., San Diego, CA) using a liquid chromatography system (Äkta Explorer 10 Chromatography System, GE Healthcare, Piscataway, NJ). The albumin-free fractions were collected and complete albumin removal was verified using silver-stained SDS-PAGE gels. Fractions were pooled and concentrated by ultrafiltration (Centriprep Ultracel YM-10, Millipore Corp, Bedford, MA). Protein concentration was determined by the Bradford method (Coomassie Protein Assay Reagent, Pierce Chemical, Rockford, IL) and samples were frozen at -80^{o}C until analysis.

Two-dimensional differential in-gel electrophoresis (2-D DIGE)

Protein was precipitated with acetone and dissolved in DIGE labeling buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, pH 8.5). Samples were divided into duplicate aliquots and fluorescently labeled by combining 50 μg of protein with 200 pmol CyDye DIGE Fluors (GE Healthcare). One aliquot was labeled with Cy3 while the other was labeled with Cy5 in case preferential labeling with the dyes occurred. A pooled sample containing equal amounts of each sample was labeled with Cy2. The labeling reactions were quenched with 10 mM lysine. The samples were randomly mixed so that one Cy3 and one Cy5-labeled sample were loaded on a single gel, along with the Cy2-labeled pooled sample, which was used as an internal standard and allowed for each resolved protein to be semi-quantitatively assessed relative to the standard

within and between each gel, thereby minimizing gel-to-gel variation. Samples were adjusted to contain 1 mg/ml DTT, and the labeled proteins (350 µg) were loaded onto immobilized pH gradient (IPG) strips (13 cm, pH 4-7, Immobiline™ DryStrip, GE Healthcare) by passive diffusion (GE Healthcare). Isoelectric focusing was performed on an IPGPhor (GE Healthcare) according to the manufacturer's instructions (500 volts for one hour followed by 1000 volts for one hour followed by a linear gradient to 8000 volts until approximately 35,000 Vhr). The focused strips were equilibrated in two steps: 15 minutes in SDS equilibration buffer I (6M urea, 2% SDS, 30% glycerol, 50 mM Tris, pH 8.8, 0.01% bromophenol blue, and 10 mg/ml DTT) followed by 15 minutes with equilibration buffer II in which the DTT was replaced by 25 mg/ml iodoacetamide. The equilibrated IPG strips were placed directly on top of polymerized 12% SDS gels[139] and covered with low-melt agarose. Gels were run in cooled tanks at 600V, 35 mA/gel for several hours until the dye front had run off the bottom of the gel.

Gel image analysis

Gel images were obtained using a Typhoon™ Trio, Variable Mode Imager (GE Healthcare). DeCyder software (version 6.5, GE Healthcare) was used to detect spots, subtract background, and to normalize spots against the pooled standard, match spots between gels and determine significant changes in abundance ($p < 0.1$). Spot detection and matching were verified visually. Gels to be used for spot picking were fixed in 10% methanol and 7.5% acetic acid overnight, stained with Deep Purple™ Total Protein Stain (GE Healthcare) and imaged on the Typhoon. The post-stained spots were matched

to the CyDye gel images using DeCyder software. Picking and digestion were performed using Ettan robotic components (GE Healthcare).

Protein identification

Spots that showed significant differences in abundance between TP 1 and TP 2 were robotically picked, washed, and digested with recombinant porcine trypsin (Promega) as described.[140] Extracted tryptic peptides were concentrated by SpeedVac and analyzed by nano-electrospray ionization/ion trap mass spectrometry (LC/MS/MS with an LCQ Deca XP 3D ion trap and/or an LTQ linear ion trap (ThermoFinnigan, San Jose, CA)).

Protein identification was performed using both TurboSequest and MASCOT search engines. The TurboSequest analysis software (Bioworks version 3.1, ThermoFinnigan) was used to identify the peptide sequences from a subset of sequences obtained from the NCBI protein database (Release July 2007) that was prepared using search terms “canine, canis, and dog”. DTA files were generated for each MS/MS spectrum with a minimum ion count of 8 from the raw data using default parameters for the peptide mass range of 0-3500. Peptide (parent ion) tolerance of 10 ppm, fragment ion tolerance of 1.5 Da, and 2 missed cleavages for trypsin were allowed. Carbamidomethylation on Cys (+57 Da) was set as a fixed modification and oxidation on Met (+16 Da) as a variable modification. The following criteria were used for filtering peptides with low confidence scores: cross-correlation values (Xcorr) greater than 1.5 for singly charged ions, 2.0 for doubly charged, and 3.0 for triply charged ions, respectively. Any protein with two unique peptides identified was considered a match.

The MASCOT program (v2.2, Matrix Science, Boston, MA) was used to search the canine genome (released 2005 from NCBI) with the following parameters: one missed cleavage by trypsin, monoisotopic peptide masses, peptide mass tolerance of 1.2 Da, and fragment mass tolerance of 0.8 Da. Oxidation of methionine and carbamidomethylation of cysteine were taken into consideration. Any protein identified with this program at a significance < 0.05 was considered a match.

Western blot

Because retinol binding protein (RBP) was identified as a potential marker for renal disease progression using 2-D DIGE, we extended its evaluation to 25 dogs affected with XLHN and 19 normal age-matched littermates using Western blot. Urine samples were evaluated every 2-4 wks in the affected dogs (average 10 time points/dog) and every 1-2 months in the normal dogs (4 time points/dog). Urine samples were normalized to 20 mg/dl creatinine and loaded on duplicate 15% SDS-polyacrylamide gels (Ready Gel Tris-HCl Gel, BioRad, Hercules, CA). Purified human retinol binding protein (0.05 µg/lane, Sigma, St. Louis, MO) was loaded on each gel in order to serve as a positive control as well as to allow for semi-quantitation of RBP in each urine sample. Protein was transferred to a nitrocellulose membrane, and membranes were blocked for 2 hrs with a 6% milk solution, incubated for 1 hr at room temperature with polyclonal rabbit anti-human retinol binding protein antibody (1:4,000, Dako, Carpinteria, CA), washed for 15 min, and incubated for 1 hr with a HRP-labeled secondary antibody (goat anti-rabbit, 1:10,000, Dako). Signal was detected using an enhanced chemiluminescent

substrate (Pierce ECL Western Blotting Substrate, Thermo Scientific, Rockford, IL). Films were scanned (Epson scanner) and band density was quantified with ImageJ.[141]

Surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF)

For SELDI-TOF analysis, there are 6 different chromatographic chip surfaces available, and 3 of these chip types were chosen for analysis in this study based on preliminary experiments: cationic exchange (CM10), immobilized metal affinity capture (IMAC30), and reverse phase (H50) chips (Bio-Rad). CM10 spot surfaces contain carboxylate groups, providing an anionic surface that can interact with positively charged amino acid groups. IMAC30 spot surfaces are covered with nitrilotriacetic acid groups that form stable complexes with polyvalent metal ions, and after binding to a metal, these complexes interact with specific amino acids, such as histidine, cysteine, and tryptophan. H50 spot surfaces contain methylene group chains that bind hydrophobic amino acids.[142] Chip conditions were optimized for protein concentration, sample to buffer ratio, and matrix type and concentration. Instrument conditions were optimized for laser intensity. The matrices used were sinapinic acid (SPA) and α-cyano-4-hydroxycinnamic acid (CHCA). Preliminary analysis was performed for CM10 using both SPA and CHCA, IMAC30 using SPA, and H50 using both SPA and CHCA. Results appeared promising for all combinations except H50 using the SPA matrix. Albumin-free samples described above were diluted to a protein concentration of 1.2 μg/μl. Urea (9M urea, 2% CHAPS) was added to the samples in a 2.5:1 sample to urea buffer ratio, and the mixture was vortexed for 30 minutes.

Chip preparation

IMAC30 and H50 chips required pretreatment before addition of binding buffer. IMAC30 spots were first incubated with 5 µl charging solution (0.1 M copper sulfate) for 10 minutes, followed by incubation with 5 µl neutralization buffer for 5 minutes (0.1 M sodium acetate, pH 4). Both steps were followed by washing with 5 µl HPLC-grade water for 1 minute. H50 spots were incubated with 5 µl 50% acetonitrile for 5 minutes, and repeated once, after which the array was allowed to dry for at least 30 minutes. All chips were then incubated with 5 µl of their respective binding buffer (5 minutes for CM10 and IMAC30, 2 minutes for H50), repeated once. Binding buffers used were 0.1 M sodium acetate, pH 4 for CM10 chips; 0.1 M sodium phosphate, 0.5 M NaCl, pH 7 for IMAC 30 chips; and 10% acetonitrile, 0.1% trifluoroacetic acid for H50 chips. All buffers used were provided by the manufacturer (Bio-Rad). Urine samples were applied to duplicate spots and two quality control samples were run in quadruplicate. Samples were applied (5 µl) to spots in the following sample to binding buffer ratios: 1:4 for CM10-SPA and H50-CHCA; 2:1 for CM10-CHCA; and sample only (no binding buffer) for IMAC-SPA. The chips were incubated for 30 minutes in a humid chamber at room temperature. The sample was removed, and chips were washed three times in their respective binding buffer for 5 minutes using a 15 ml conical tube. Chips were then washed twice in HPLC-grade water for 1 minute and allowed to air-dry for 20 minutes. Matrix (either 1 µl 100% SPA or 0.5 µl 50% CHCA, both dissolved in 0.5% trifluoroacetic acid and 50% acetonitrile) was added twice to each spot, with air-drying

for 10 min between applications. Chips were allowed to dry for at least 1 hour before analysis.

Chip analysis

Chips were inserted into a ProteinChip SELDI reader (Personal edition, Bio-Rad), and the spot surface was impacted by a focused laser. This resulted in desorption of the protein from the chip surface and the flight of positively charged ions to the detector surface, where time-of-flight mass spectra were collected. A laser intensity of 1800 nJ and 3500 nJ was used for the 0-20 kDa and 20-200 kDa molecular mass ranges, respectively, using SPA matrix. A laser intensity of 1200nJ was used for the 0-15 kDa range using CHCA matrix. Two warming shots were performed at each position (not included in the data). Signal averages of 1070 laser shots (10 laser pulses at each position) were used to generate each spectrum, and alternate positions were shot for the low mass range (0-15 or 0-20 kDa) and high mass range (20-200 kDa) readings so that each position was used only once. For the low mass ranges, the matrix attenuation and focus mass were set at 1 kDa and 5 kDa, respectively. For the 0-200 kDa range, the matrix attenuation and focus mass were set at 5 kDa and 20 kDa, respectively. The remaining settings were left as default.

ProteinChip Data Manager (Bio-Rad) was used to analyze peaks. The baseline was subtracted using a smoothing window of 25 points and fitting width of 15 points. The spectra from each chip were normalized for total ion current, and peaks were calibrated and aligned. For spectra obtained using the SPA matrix, protein standards were used as calibrators (ProteinChip All-in-One Protein Standard II, Bio-Rad), while

for spectra obtained using the CHCA matrix, peptide standards were used (ProteinChip Peptide Standard Array, Bio-Rad).

Renal histopathology

Renal biopsies were obtained from three of the dogs when microalbuminuria was detected (11-12 weeks of age) and when serum creatinine was ≥ 1.2 mg/dl (21-23 weeks of age), which corresponded to within 1-3 weeks of time points 1 and 2, respectively, for each dog. The dogs were anesthetized (20 mg/kg thiopental) and maintained on isofluorane while ultrasound-guided needle biopsies were obtained using an 18-gauge Bard® Monopty® disposable core biopsy instrument (Bard Biopsy Systems, Tempe, AZ). Cores were immediately placed into 10% formalin and embedded in paraffin within 24 hrs. Three μm sections were cut from two levels of each biopsy 100 μm apart in order to represent a different plane of the biopsy. Sections stained with H&E, Masson's trichrome, and PAS were evaluated by a board-certified pathologist. The interstitium, tubules, and glomeruli were scored as follows. Twenty 400X fields of tubulointerstitium were evaluated in each trichrome stained section and the presence or absence of interstitial fibrosis was recorded. Twenty 400x fields of tubulointerstitium were evaluated in each H&E stained section and presence or absence of tubular epithelial cell degeneration was recorded. All glomeruli were evaluated in each PAS stained section and the presence of the following features were recorded: podocyte hypertrophy, atrophy of the glomerular tuft with dilation of Bowman's capsule, synechiae, and small and large fibrinous crescents.

Statistical analyses

Serum creatinine concentration, UPC, and urine specific gravity were analyzed with a paired sample t-test (SPSS 11, Version 11.0.4). Data appeared normally distributed for each time point. For 2-D DIGE analysis, spots were statistically analyzed using a paired sample Student's t-test and one-way ANOVA performed by the DeCyder BVA module. For SELDI-TOF, each chip type was analyzed independently using the ProteinChip Data Manager. Spectra were subjected to Expression Difference Mapping (EDM) using a 20% threshold value and a first pass of S/N and valley depth > 5 and a second pass of S/N and valley depth > 2. The paired Wilcoxon signed rank test was used to calculate p-values, determining significance between the two time points. Statistically significant peaks were scrutinized for quality of peak as well as the number of estimated peaks versus detected peaks in each peak cluster. Receiver operating curves (ROC) were also calculated. Statistical significance was set at $p < 0.05$ for all tests except for 2-D DIGE ($p < 0.1$).

Results

Clinical data

Mean age, serum creatinine concentration, and urine protein:creatinine ratio were significantly higher for TP 2 as compared to TP 1 (Table 3). However, no significant difference was observed for the mean specific gravity between the two time points.

Table 3. Comparison of clinical parameters (mean ± standard deviation) between time points 1 (early proteinuria) and 2 (early azotemia).

	Time point 1	Time point 2	p-value
Age (wks)	18.7 ± 5.7	27.2 ± 6.8	<0.001
Serum creatinine (mg/dl)	0.6 ± 0.1	1.4 ± 0.3	<0.001
Urine protein:creatinine	7.9 ± 5.0	15.1 ± 4.8	0.019
Urine specific gravity	1.016 ± 0.008	1.014 ± 0.005	0.585

Renal histopathology

Histopathological evaluation of the renal biopsies of 3 dogs was performed in order to assess the degree of tubulointerstitial and glomerular damage present at the two time points (Figure 5). In order to achieve a non-biased evaluation of the renal architecture, 20 randomly selected high magnification (400x) fields of tubulointerstitium were examined and scored for the presence or absence of interstitial fibrosis and tubular degeneration. At TP 1 both the tubules and the interstitium in all 3 dogs were normal; neither interstitial fibrosis nor tubular epithelial cell degeneration were present in any of the specimens. Evaluation of all available glomeruli at this time point revealed only podocyte hypertrophy in 20-35% of the glomeruli in each specimen. At TP 2, 60-70% of the evaluated 400x fields had mild interstitial fibrosis; 75-85% of the fields contained tubules with evidence of epithelial cell degeneration. Podocyte hypertrophy and dilation of Bowman's capsule with atrophy of the glomerular tuft were consistent lesions of almost all glomeruli examined at this time point. In addition, fibrinous crescents and adhesions between the glomerular tuft and Bowman's capsule (synechiae) were present in < 50% of the glomeruli.

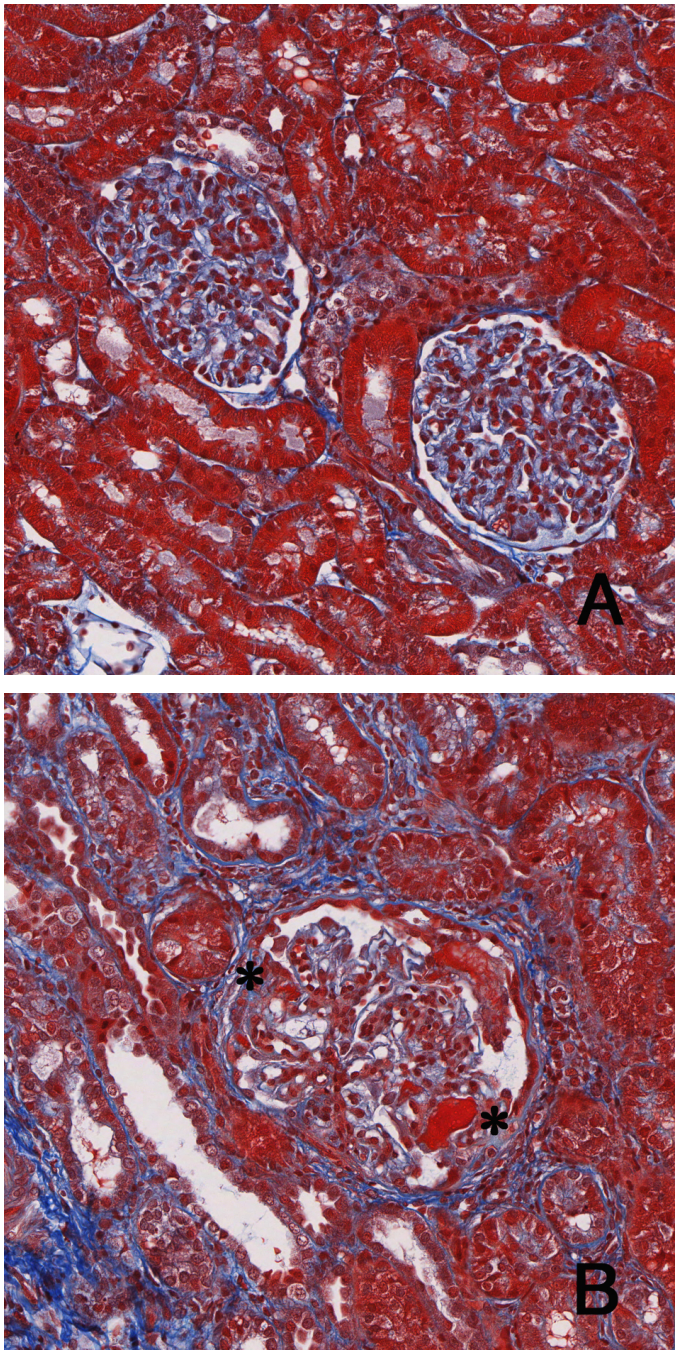

Figure 5. Photomicrographs of renal biopsies from a single dog at time points 1 and 2. At TP 1 (A, early proteinuria), the tubulointerstitium is histologically normal and mild glomerular podocyte hypertrophy is evident. At TP 2 (B, early azotemia), mild interstitial fibrosis and tubular degeneration are present, along with more pronounced glomerular changes, such as synechiae(*). Masson's trichrome, x20 objective.

2-D DIGE

A fluorescent image of a representative 2-dimensional gel showing CyDye-labeled proteins is shown in Figure 6. The numbers represent spots that were picked and identified, and correspond to those listed in Table 4. Approximately 900 protein spots were visualized on each gel, and protein spot patterns were essentially identical among the animals, as determined by spot matching and analysis using DeCyder software. A comparison of the albumin-depleted urine from the early and later time points revealed differential presence of many protein spots, of which 22 were selected for analysis. Of these, 16 spots yielded significant data by mass spectrometry and database interrogation (Table 4). Several of the identified proteins were found in multiple spots suggesting that they may represent related isoforms or protein modifications. In addition, some spots contained multiple proteins. Thus, collectively, the identified spots represented 18 different proteins. Eleven spots showed a significantly greater volume at the earlier time point, whereas 5 spots had a significantly greater volume at the later time point (Table 4). The proteins identified from the spots that were more abundant at TP 2 include retinol binding protein, hemopexin, apolipoprotein A1 (Apo A1), and perlecan. The proteins identified from the spots that were more abundant at TP 1 included gelsolin, Apo A1, complement C3, collagen type XXVII, α-2-HS-glycoprotein (Fetuin A), haptoglobin, junctional adhesion molecule A (platelet adhesion molecule 1), immunoglobulin, and AMBP protein.

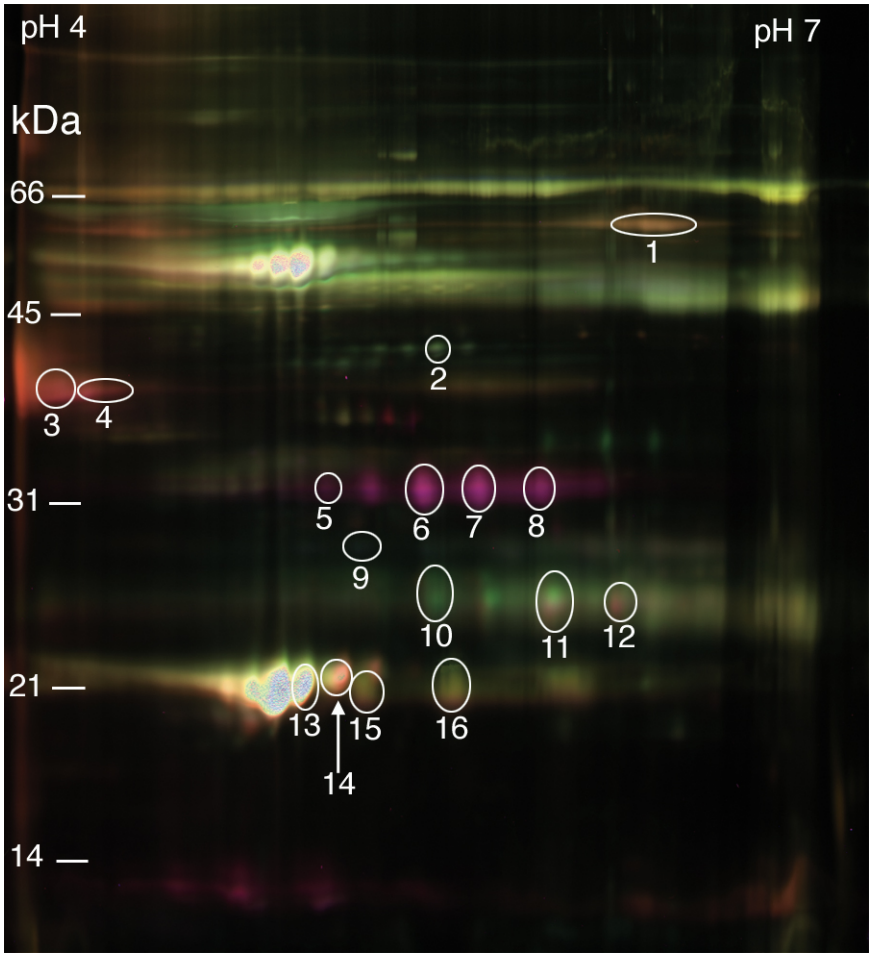

Figure 6. Scan of a gel from analysis of urine proteins separated by 2-dimensional gel electrophoresis, stained with CyDye. The outlined spots were differentially present in the urine obtained from time point 1 (early proteinuria) as compared with time point 2 (early azotemia) and were therefore picked from the gel and subjected to analysis by mass spectrometry. The numbered spots correspond to Table 4.

Table 4. Identification of proteins from corresponding spots on the 2-D DIGE gel (Figure 6) that were differentially present between time point 1 (early proteinuria) and time point 2 (early azotemia). Bold and italicized spots were increased in TP 2.

Spot Label[a]	Accession number[b]	Protein ID	Mascot (Score)	SEQUEST (Delta Cn)	p value
1	**gi|73988725**	***Hemopexin***	62	20	0.001
2	gi|73971658	Gelsolin	NI	30	0.043
	gi|73955106	Apolipoprotein A-I	NI	30	
	gi|73965965	Keratin 25A (canine)	71		
	gi|61740600	Keratin 10 (canine)	71		
3	gi|73955106	Apolipoprotein A-I	NI	100	0.048
		Collagen type XXVII	NI	60	
4	gi|73987236	Complement C3	60	86	0.056
	gi|73972000	collagen type XXVII	59	50	
	gi|74003450	α-2-HS-glycoprotein (Fetuin A)	58	40	
	gi|50979272	Epithelial keratin (canine)	48	NI	
	gi|74003125	Solute Carrier family 12	45	NI	
	gi|73956752	Ubiquitination factor	42	NI	
	gi|73971743	Aquaporin-7	39	NI	
5	gi|73957095	Haptoglobin	NI	30	0.08
6	gi|73957095	Haptoglobin	84	58	0.077
7	gi|73955106	Apolipoprotein A-I	43	70	0.074
	gi|73957095	Haptoglobin	NI	20	
8	gi|73957095	Haptoglobin	NI	70	0.087
9	gi|74006287	Junctional adhesion molecule A	72	NI	<0.001
	gi|73995629	Immunoglobulin	NI	30	
10	gi|73980858 gi|74012534	Immunoglobulin	66	30	<0.001
11	gi|73980864	Immunoglobulin	113	50	0.007
	gi|73971996	AMBP protein	41	20	
	gi|73959812	GPI-anchor transamidase	39	NI	
12	gi|73980864	Immunoglobulin	78	40	0.008
13	**gi|73955106**	***Apolipoprotein A-I***	64	370	0.038
	gi|73950646	***Perlecan***	NI	20	
14	**gi|73955106**	***Apolipoprotein A-I***	62	270	0.001
15	**gi|73998292**	***Retinol binding protein***	48	NI	<0.001
16	**gi|73998292**	***Retinol binding protein***	84	NI	<0.001
	gi|73959812	***GPI-anchor transamidase***	40	NI	

[a]Spot label number from annotated gel image
[b]NCBI (gi) protein database accession numbers
NI, Not identified

Western blot confirmed the increase in RBP seen with 2-D DIGE analysis. On average, RBP first became detectable at 19 weeks of age in affected dogs (approximately 2 months before creatinine > 1.2 mg/dl) and increased with progression of disease (Figure 7). RBP was not detected by Western blot in any of the normal dogs.

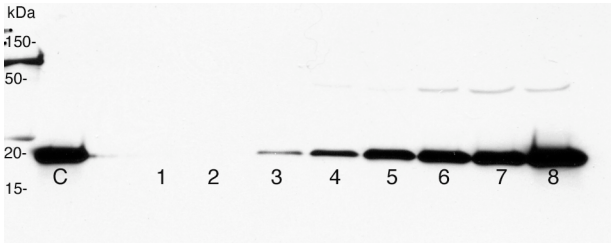

Figure 7. Western blot for retinol binding protein (RBP) in urine samples from a male dog with XLHN from 10 wks of age (Lane 1) to 26 wks of age (Lane 8), revealing an increase in urinary RBP with renal disease progression. Purified human RBP (0.05 µg) was loaded in the lane labeled "C". Polyclonal rabbit anti-human retinol binding protein was used as the primary antibody, and HRP-labeled goat anti-rabbit antibody was used as the secondary antibody. Signal was detected using an enhanced chemiluminescent substrate.

SELDI-TOF

The peaks obtained with the CM10 and IMAC30 chips using SPA matrix showed good peak reproducibility in the quality control samples based on coefficient of variation (CV) (peak intensity CV < 30%). Also, the peaks obtained with the H50 chip using CHCA matrix produced acceptable peak reproducibility (typically peak intensity CV < 30%). However, peaks obtained with the CM10 chip using CHCA matrix showed poor reproducibility and were therefore excluded from analysis. Up to 172 peaks were

detected on a single chip type, and more peaks were identified in the low mass range (2-20 kDa) than the high mass range (20-200 kDa), supporting the utility of SELDI-TOF to detect small proteins and protein fragments (Table 5).

Table 5. Summary of the number of peaks obtained using SELDI-TOF for CM10, IMAC30, and H50 chips

	CM10		**IMAC30**		**H50**
Mass range (kDa)	0-20	20-200	0-20	20-200	0-15
Total number of peaks detected	64	34	129	43	44
Number of peaks significantly different between time points	33	20	41	13	15
Number of peaks higher at TP1	19	2	21	4	8
Number of peaks higher at TP2	14	18	20	9	7

Among all chips, there were 93 unique, well-defined peaks that demonstrated a statistically significant difference in peak intensity between the two time points. Several of these peaks were identified using more than 1 chip surface, with CM10 and IMAC30 most commonly identifying the same peak. Those peaks that accurately identified a particular time point in each dog (ROC = 0 or 1) and that were either present on more than one chip type or that had a particularly high level of induction within a single chip are listed in Table 6. Representative spectra from each chip are shown in Figure 8. The increased or decreased intensity of the peaks is illustrated in Figure 9 for the CM10 chip, showing higher peak intensity at TP 1 for the majority of peaks in the 2-20 molecular mass range versus higher peak intensity at TP 2 for the majority of peaks in the 20-200 molecular mass range. All peaks > 50 kDa were of greater abundance at TP 2.

Table 6. Listing of peaks identified using SELDI-TOF that are both well-defined and that accurately identify the time point in all dogs based on ROC analysis. Peak mass, the chip on which the peak was identified, and the time point in which the peak was observed to be higher are listed.

Peak mass (Da)	**Chip present**	**Time point**
2778	CM10	TP1
3224	CM10	TP1
4073	CM10, IMAC30	TP1
4142	IMAC30	TP1
4393	CM10, IMAC30, H50	TP2
4539	IMAC30	TP2
4726	CM10, IMAC30	TP2
4795	IMAC30	TP1
4930	CM10	TP1
4966	CM10, IMAC30	TP2
5021	CM10, IMAC30	TP1
5045	IMAC30	TP2
5401	CM10	TP1
5543	CM10	TP1
5758	CM10, IMAC30	TP2
7424	IMAC30	TP1
8549	CM10, IMAC30, H50	TP2
9665	CM10, IMAC30, H50	TP1
9828	CM10, IMAC30, H50	TP1
9950	IMAC30	TP2
10,379	CM10, IMAC30, H50	TP1
10,909	IMAC30	TP1
11,312	CM10, IMAC30	TP2
11,505	CM10, IMAC30	TP2
13,017	IMAC30	TP2
13,731	CM10, IMAC30, H50	TP2
15,214	CM10, IMAC30	TP1
20,572	CM10, IMAC30	TP1
20,957	CM10	TP2
23,152	CM10	TP2
39,063	CM10	TP2
59,384	IMAC30	TP2
77,729	CM10	TP2
103,962	CM10, IMAC30	TP2

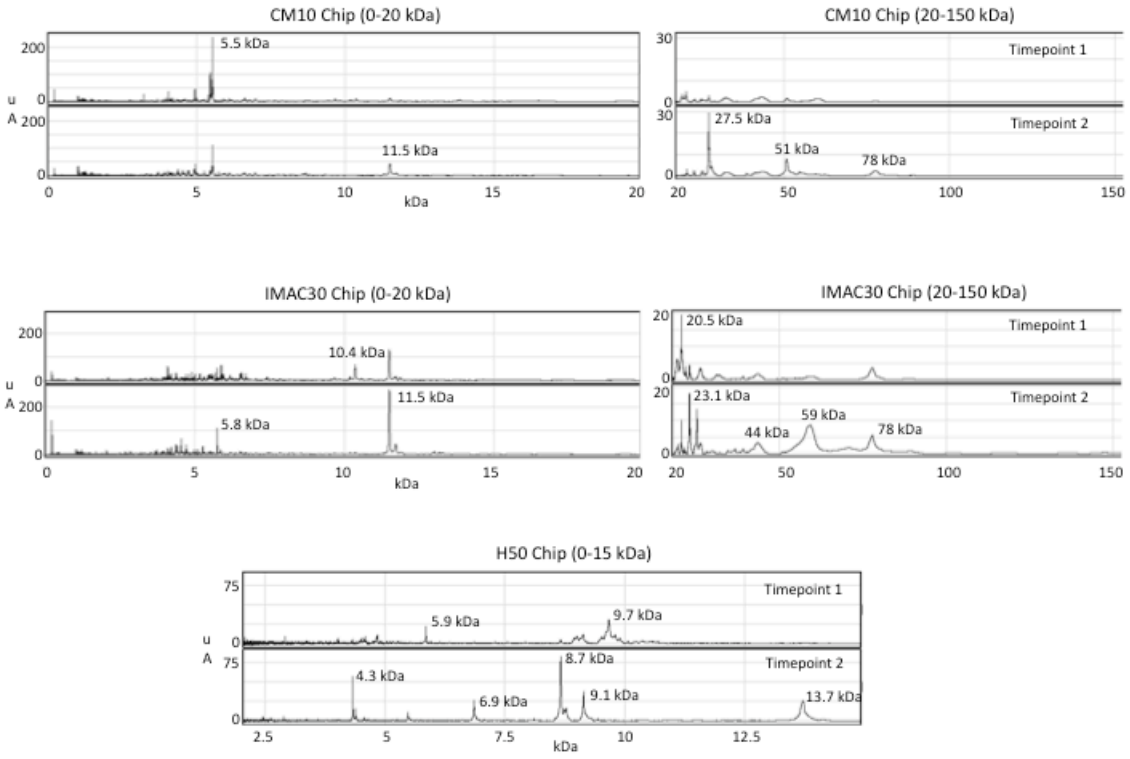

Figure 8. Representative spectra obtained from CM10, IMAC30, and H50 chips from a single dog at time point 1 as compared with time point 2. Spectra show differences in many peaks between the two time points and among chip types. The peaks range in molecular weight from 0-150 kDa (CM10 and IMAC30 chips) and 0-15 kDa (H50 chip).

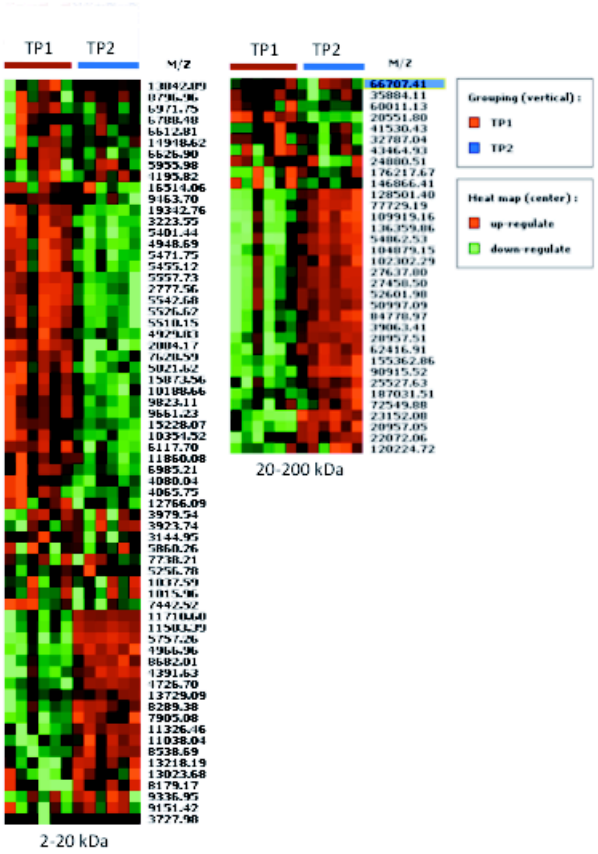

Figure 9. Heat map plot of the peaks identified on the CM10 chip within the 2-20 kDa range and the 20-200 kDa range. The areas of green indicate decreased peak intensity whereas the areas of red indicate increased peak intensity. Increased peaks at the first time point (TP 1) are mostly in the 2-20 kDa range whereas increased peaks during the second time point (TP 2) are mostly in the range of 20-200 kDa.

Discussion

In an effort to discover novel renal biomarkers and to gain insight into the molecular mechanisms underlying the pathogenesis of canine tubulointerstitial injury, we performed a comparative analysis of the urine protein profiles in dogs during early stages of CKD caused by XLHN using 2-D DIGE and SELDI-TOF. These techniques were both successfully employed in this study to identify proteins in the urine that are

altered with renal disease progression. With 2-D DIGE, the spot volume was compared between two stages of renal disease progression, and proteins within significantly increased or decreased spots were identified. With SELDI-TOF, the peak intensity of proteins bound to different chromatographic surfaces was compared between time points.

The differentially present proteins identified in this study may be altered with disease progression due to several mechanisms. In normal dogs, the majority of proteins < 40 kDa freely pass through the glomerular filtration barrier along with a small amount of albumin, and these low molecular weight (LMW) proteins are nearly completely reabsorbed by the renal tubules, mediated primarily by the receptors megalin and cubilin, so that very little protein is present in normal urine.[57] In dogs with CKD due to glomerular disease, massive proteinuria often results, with subsequent passage of intermediate molecular weight (IMW) and high molecular weight (HMW) proteins from the plasma into the urine filtrate. LMW proteins that otherwise would normally be reabsorbed by the tubules appear in the urine secondary to decreased tubular reabsorption, which is likely due to a combination of increased competition for binding sites on megalin and cubilin (due to the presence of IMW and HMW proteins) and decreased numbers or function of these receptors (due to tubular damage).[104] In addition, proteins can be released from damaged tubules. In the present study, mean UPC was significantly higher at TP 2 as compared to TP 1; however, marked proteinuria and subsequent increased competition for reabsorption was present at both time points (Table 3). While glomerular damage worsened with disease progression, resulting in a higher

UPC and additional competition for reabsorption, it is likely that tubular damage and/or dysfunction is a major contributing factor for the observed increase in many of the LMW proteins. This is supported by the development of azotemia and the histopathologic findings of increased interstitial fibrosis and tubular degeneration in biopsies taken during the later time point (Figure 5). In addition, one study using male dogs with XLHN demonstrated decreased reabsorption by renal tubules with disease progression, which corresponded to increased urinary excretion of several LMW proteins and altered protein handling within the proximal tubular cells.[104] These findings support the rationale for using the chosen time points in this canine model of CKD to discover novel biomarkers of early tubulointerstitial injury.

The 2-D DIGE analysis revealed a number of proteins that were differentially present in the urine samples from the two time points, and the majority of proteins identified were present in spots that were more abundant during the first time point. Several of the identified proteins, including retinol binding protein, hemopexin, AMBP, Fetuin A, complement C3, gelsolin, and perlecan have all previously been implicated in renal disease or are involved in renal pathophysiology.[27,143-150] For instance, AMBP is proteolytically processed into α1-microglobulin (α1m) and bikunin, and urinary α1m has been extensively studied as a biomarker of tubulointerstitial disease in people.[27] In human patients with CKD, plasma gelsolin levels decreased with progression of disease, and low plasma gelsolin levels have been correlated with increased mortality in patients receiving hemodialysis.[144] Inflammatory cytokines may induce hemopexin production in mesangial cells,[145] and hemopexin infusion in rats results in proteinuria and podocyte

foot process effacement.[146] The present study is the first report linking many of these proteins with renal disease in dogs, and further evaluation is needed to determine their significance.

The most studied of the identified proteins is RBP, which was more abundant in the later time point. RBP has been evaluated as a marker for tubulointerstitial damage and an indicator of prognosis in humans with renal disease,[85,143] and there are several reports in veterinary medicine that demonstrate its relative abundance in urine samples from dogs with renal disease as compared with normal dogs, although serial evaluation was not performed.[102,103,107,114] In the present study, increased urinary RBP was verified by Western blot in 25 XLHN dogs, revealing a progressive increase during renal disease progression (Figure 7). In addition, RBP was detected in the urine of these dogs well before the development of azotemia, supporting its use in detecting early tubulointerstitial disease in addition to progression of disease. While the role and behavior of the other identified proteins in dogs with renal disease is unknown, the identification of a known urinary biomarker of renal disease in this study supports the use of 2-D DIGE for the discovery of novel renal biomarkers.

As might be expected with a global proteomic study, several unexplained observations were made. Apo A1 was present in some spots that had increased abundance and others with decreased abundance at the later time point. This could be consistent with the presence of different isoforms or modifications of the protein, and perhaps measurement of different forms of the protein in urine would be useful in renal disease; however further evaluation of Apo A1 in both the serum and urine of dogs with

CKD would be necessary to assess this possibility. In addition, immunoglobulin was identified only in spots with decreased abundance at the later time point. This is unexpected, as immunoglobulins should more easily pass through the glomerular filtration barrier due to more severe glomerular damage at the later time point. With SELDI-TOF analysis, there was a peak identified at 155 kDa on the CM10 chip, which may correspond to IgG, and this peak was higher at TP 2 in all dogs. In addition, measurement of urinary IgG by ELISA in these dogs demonstrated an increase in IgG in the early stages of disease progression as described in Chapter IV. The discrepancy of these findings with the 2-D DIGE results is likely a reflection of one of the weaknesses of 2-D electrophoresis, where components of a protein may be analyzed separately and may not reflect the behavior of the intact protein.

Another challenging area with 2-D electrophoresis utilizing mass spectrometry-based methods of protein identification is how to ensure confidence in the protein identity, particularly with differing database interrogations. For instance, in Spot 4, several proteins were identified using Mascot, but they had a relatively low Mascot score and were only identified with a single search engine. These proteins were included in Table 4 since they obtained a significant score. However, clusterin was identified with 3 unique peptides in Spot 4 using TurboSequest, but it was not included in Table 4 since only one of the peptides met the necessary minimum Xcorr value. Clusterin expression is upregulated in renal injury due to a variety of causes in people[151] and mRNA levels of clusterin were upregulated in end-stage kidney disease in XLHN dogs.[152] Therefore, clusterin would be an interesting protein to evaluate further in dogs with CKD. These

results illustrate the differences seen with the use of different databases and different programs to search MS/MS spectra. For those spots where both searches revealed the same protein identification, high confidence can be placed in that identification. However, for those spots where some proteins were identified with only one program, results may be questionable, particularly when numerous proteins with a low score were identified within the same spot. These findings illustrate that 2-dimensional gel electrophoresis, while useful as a discovery tool, must be confirmed independently with more specific techniques. In the present study, one of the proteins identified (RBP) was confirmed with Western blot. Lack of cross-reactive antibodies prevented investigation of additional promising proteins.

To complement the 2-D DIGE results, SELDI-TOF analysis of the samples was performed. Although SELDI-TOF does not allow for protein identification without substantial further analysis, it does provide a global view of the size of proteins present in the urine, and it is particularly useful for detecting proteins and protein fragments < 20 kDa. In this study, spectra from TP 2 samples had a higher number of significantly increased peaks as compared to TP 1 for two of the chip types, but it is important to note that there was a mixture of peaks that were increased in either samples from TP 1 or TP 2, similar to results from the 2-D DIGE analysis. All peaks > 50 kDa were more abundant at TP 2 than at TP 1, and most peaks > 20 kDa were more abundant in TP 2. The peaks < 50-60 kDa that were of higher abundance during TP 2 likely represent worsening tubular damage, whereas those peaks > 50-60 kDa that were of higher abundance during TP 2 likely represent worsening glomerular damage. Proteins < 20

kDa were more often decreased in TP 2 (Table 5, Figure 9), and this may reflect a decrease in relative abundance with respect to the total amount of protein present in TP 2, as other proteins increase with disease progression. An alternative explanation for the decrease of these peaks during TP 2 as compared to TP 1 may be that these are proteins present in normal urine and excretion is decreased with tubular damage. Since the objective of this study was to evaluate markers of early tubulointerstitial disease that appear during renal disease progression, urine from normal dogs was not evaluated. However, urine from normal dogs does contain a small number of well-defined LMW peaks when analyzed with SELDI-TOF (unpublished observations), and in normal human urine, many LMW peaks are found.[153]

The SELDI-TOF results identified many well-defined, differentially present peaks that might serve as useful biomarkers of renal disease, either alone or in combination (Table 6). Of particular interest are the statistically significant peaks present on multiple chips and those present on a single chip with a high level of induction, and these include peaks at: 3.2, 4.5, 4.8, 5.0, 5.4, 5.8, 8.5, 10.4, 10.9, 11.5, 13.0, 20.6, 21, 27.5, 39.0, 59.4, 77.7, and 104 kDa. Some of the identified peaks illustrate that SELDI-TOF can be useful in detecting protein modifications that are present at different stages of disease. For instance, the peak at 5,021.9 Da was increased at TP 1, whereas the peak at 5,045.4 Da was increased at TP 2 in each dog. This ~24 Da difference could represent a modification of the same protein rather than two different proteins.

Only one other study has used SELDI-TOF in the analysis of urine from dogs with renal disease, and in this study, far fewer peaks were identified as being altered in

dogs with renal disease as compared with normal dogs.[107] The larger number of peaks identified in the present study may be secondary to enrichment of low abundance proteins due to albumin removal or because paired samples were used from dogs with fewer confounding factors. The peaks identified by Forterre, et al were 11.6, 12.4, 12.6, 14.6, 21, 27.9, and 65.7 kDa, all of which were significantly increased in dogs with renal disease except the 27.9 kDa peak, which was increased in normal dogs as compared with diseased dogs.[107] This study also noted that in dogs with renal disease, the peak corresponding to 27.9 kDa was actually ~27.5 kDa, suggesting a modification of this protein.[107] Of these peaks, the present study identified three (~11.5, 21, and 27.5 kDa), all of which were increased at TP 2. The peak at 27.5 kDa corresponds to the molecular mass of this peak in those dogs with renal disease in the previous study. The ~66 kDa protein likely represented albumin, which was removed from the present study. The identification of proteins of nearly identical molecular mass in two separate studies supports the use of SELDI-TOF for accurate peak detection in canine urine.

It is important to note that while proteins cannot be reliably identified based solely on their molecular mass, there are several peaks that have a mass similar to known biomarkers of renal disease that were found to be differentially present with SELDI-TOF analysis. For example, the peak at 11.5 kDa might represent ß2-microglobulin and the peak at 21 kDa might represent RBP. As would be expected for these proteins, both of these peaks were elevated at TP 2. In addition, RBP was verified to increase with disease progression in the present study.

Although this study comprehensively evaluates the urine proteome in dogs with early stages of chronic renal disease, there are several limitations. First, albumin was removed from the samples analyzed. While this helped to enrich for less abundant proteins, important biomarkers that bind to albumin may also have been removed. In addition, other highly abundant proteins in the plasma were detected in multiple spots with 2-D DIGE (e.g., immunoglobulin, haptoglobin, and Apo A1) supporting their abundance in canine plasma and in the urine of dogs with glomerular disease. Additional studies comparing samples without albumin removal and with both albumin removal and removal of other abundant proteins would be helpful to assess the effects of these proteins on the detection of urinary biomarkers and to further enrich for low abundant renal biomarkers. Second, dogs were in the puppy to adolescent stage between the two time points, and without the inclusion of normal dogs, aging-related changes in the urine proteome cannot be separated from changes due to renal disease progression. However, this is thought to be a minor limitation, as any aging-related changes in the urine proteome are likely to be overwhelmed by the marked proteinuria present in these dogs. It is also important to keep in mind that this study was performed in dogs with a single disease process, and while its clinical and histologic progression is similar (although more rapid) to CKD caused by a variety of glomerular diseases, the findings need to be verified in a more diverse population of dogs with CKD due to multiple causes.

Lastly, each technique has its own limitations. With the 2D-DIGE, there were a number of highly significant spots that could not be identified by mass spectrometry because of their low abundance. In addition, this technique is not ideal for detecting

proteins that are hydrophobic, highly acidic, highly alkaline, or poorly soluble. With SELDI-TOF, a large number of well-defined peaks were detected, but the identity of most of the peaks remains unknown without extensive follow-up.

In summary, this study revealed a number of proteins that are differentially present in early stages of tubulointerstitial disease in male dogs with X-linked hereditary nephropathy. Several of these proteins are well-established as markers of renal disease in humans and are in the early stages of evaluation in dogs with renal disease. In addition, this study has implicated several additional proteins as markers of canine tubulointerstitial injury for the first time.

CHAPTER IV

URINARY BIOMARKERS OF RENAL DISEASE IN DOGS WITH CHRONIC RENAL FAILURE DUE TO NATURALLY-OCCURRING PROGRESSIVE GLOMERULAR DISEASE

Overview

Current non-invasive diagnostic tests for renal disease in veterinary patients lack adequate sensitivity and specificity. One promising area of study that may address this issue is the evaluation of urinary biomarkers. A variety of urinary proteins have shown promise in human medicine for providing information about disease severity and progression above and beyond that obtained from using standard measures of kidney function, including serum creatinine. In veterinary medicine, these proteins have only recently been explored, and currently no study concerning the serial evaluation of urinary biomarkers in dogs with progressive renal disease is available that would allow for the correlation of these proteins with conventional clinical and histologic measures of renal function.

This study evaluates four urinary biomarkers of tubular function and/or damage and one urinary biomarker of glomerular injury in dogs with X-linked hereditary nephropathy and normal age-matched littermates. Results are correlated with serum creatinine, glomerular filtration rate, urine protein:creatinine ratio, and histologic analysis of renal biopsies. All biomarkers were able to distinguish affected dogs from normal dogs early in their disease process. However, only retinol binding protein

correlated strongly with all measures of disease severity, including increasing serum creatinine, decreasing glomerular filtration rate, and presence of irreversible histologic tubulointerstitial lesions. Therefore, measurement of this protein in urine may be promising for the diagnosis and monitoring of kidney disease in dogs.

Introduction

Chronic progressive kidney disease (CKD) leading to renal failure is a common cause of morbidity and mortality in dogs, and it is commonly a result of primary glomerular disease.[3,4] Regardless of the initiating cause of kidney disease, progressive renal injury is characterized by interstitial fibrosis, peritubular capillary loss, and destruction of functional nephrons, and renal function most closely correlates with the degree of tubulointerstitial damage.[5] However, most currently available, non-invasive clinical methods for detecting tubulointerstitial disease are relatively insensitive. In addition, specificity of these tests, particularly specific gravity, can be quite poor. There is currently no clinically available, sensitive and specific non-invasive marker to detect ongoing tubular damage and decreased tubular function that will ultimately lead to end-stage renal disease. Therefore, although tubulointerstitial lesions are frequent in dogs,[1-3] they are recognized clinically at an advanced stage, when lesions are both severe and irreversible, in which case options for successful therapy are limited. Detection of tubulointerstitial damage and altered function at an earlier stage would permit earlier interventions with renoprotective therapies that slow renal disease progression and therefore prolong survival.

In the human literature, evaluation of certain urinary proteins (i.e. qualitative assessment of proteinuria) has shown promise in determining the localization and severity of renal damage in patients with various forms of chronic renal disease. In addition, these proteins have detected tubular dysfunction and injury earlier than conventional methods. For example, low molecular weight proteins have been used to assess the degree of tubular function and therefore tubulointerstitial damage, whereby their increase in the urine is due to decreased reabsorption by the renal tubules. β2-microglobulin (B2M), α1-microglobulin (α1M), and retinol binding protein (RBP) have all been shown to provide prognostic information and to predict the clinical course of disease better than the magnitude of proteinuria, serum creatinine concentration, and in some cases renal biopsy analysis.[78-80,84,85] Other proteins evaluated include those released from injured tubular cells, such as N-acetyl-β-D-glucosaminidase (NAG), and many of these tubular proteins have also been found to be more sensitive for renal dysfunction than serum creatinine concentration or total urinary protein loss and to be superior in predicting renal disease progression and response to therapy in patients with several glomerular diseases.[90,91]

In veterinary medicine, these proteins have undergone limited evaluation. In dogs with CKD, specific protein evaluation has revealed decreases in urinary excretion of Tamm-Horsfall protein (THP), as well as increases in a variety of proteins including NAG, γ-glutamyltransferase, RBP, α1M, B2M, lysozyme, vitamin D-binding protein, transthyretin, transferrin, and immunoglobulin (IgG).[101-107] However, the temporal behavior of these urinary biomarkers in naturally occurring progressive renal disease is

unknown, as no serial evaluation has been performed in dogs comparing them with standard measures of renal function. Thus, the utility of these markers for detecting CKD earlier than standard measures and whether these markers can help identify progression of renal disease is unknown.

Therefore, the primary objectives of this study were: 1) to serially evaluate urinary biomarkers in dogs with a single chronic progressive kidney disease and compare their behavior with standard measures of renal function and damage; and 2) to assess the ability of each one of these urinary biomarkers to provide additional information beyond that obtained with other markers in order to suggest a panel of markers that may be most useful in monitoring renal disease progression. Secondary objectives were to assess assay performance of each quantitative assay and to investigate stability of NAG in canine urine.

The urinary biomarkers selected for this study were chosen based on their established use as specific markers of tubulointerstitial damage or function in the human literature, their proposed handling by the renal tubules so that multiple mechanistic processes are represented, and the availability of a commercially available antibody and/or assay. Therefore, the urinary biomarkers evaluated include those that originate from damaged tubular cells (i.e., NAG and neutrophil gelatinase-associated lipocalin (NGAL)) and those that originate from filtered plasma and appear in the urine secondary to decreased reabsorption due to tubular injury (i.e., RBP and B2M). In addition, IgG concentration in the urine (uIgG) was measured in order to assess the degree of glomerular permselectivity, an indication of the severity of glomerular lesions.

These proteins were measured in dogs with X-linked hereditary nephropathy (XLHN), a naturally-occurring glomerular disease that results in progressive CKD. Although the genetic mutation causing this disease is rare, the pathogenic pathway for developing end-stage renal disease (ESRD) in these dogs is similar to that in other dogs with glomerular disease leading to ESRD. Because of the controlled environment and extensive monitoring throughout their disease, the dogs with this specific disease provide an attractive model for studying the behavior of these urinary biomarkers in dogs with CKD secondary to primary glomerular disease in order to determine their clinical usefulness for early detection and monitoring the progression of tubulointerstitial injury and declining renal function.

Materials and methods

Dogs

For the purpose of this study, we performed analyses on stored urine samples that were collected between August, 2002 and October, 2008 from 25 male dogs with XLHN (22 intact and 3 neutered at 14 wks of age) and 19 normal male littermates (16 intact and 3 neutered at 14 wks of age). The dogs were members of a single family maintained in a colony of dogs at Texas A&M University since 1997. In this kindred, XLHN is caused by a nonsense mutation in the *COL4A5* gene that encodes the α5 chain of type IV collagen, which is a crucial component of normal glomerular basement membranes (GBM).[45] The salient clinical and pathologic features of the nephropathy that occurs in

male dogs with this gene defect have been described and include absence of the normal collagen IV network in the GBM, development of marked proteinuria, and progressive CKD that typically results in end-stage renal failure at about 1 year of age.[44]

All puppies produced in the colony were raised using a standardized protocol for feeding, husbandry, routine health care, and socialization. During studies, dogs were housed in runs in a temperature-controlled room with a 12-hour light-dark cycle, and they were fed once daily in the afternoon. Dogs were leash walked outside or were permitted short periods of unrestricted access to an exercise area daily. No treatments were administered to the dogs whose samples were utilized in this study. The study protocols were reviewed and approved by the Texas A&M University Laboratory Animal Care Committee.

Sample collection

Blood and voided, mid-stream urine were collected in the morning on a weekly to biweekly basis from the dogs starting at 8 weeks of age. Blood was centrifuged for plasma collection, and serum creatinine (sCr) and albumin were measured (Vitros 250, Johnson & Johnson Co., Rochester, NY). Urine specific gravity was measured with a refractometer and a routine dipstick analysis and semi-quantitative microalbuminuria dipstick were performed (Multistix, Bayer Corp, Elkhart, IN; ERD-HealthScreen, Heska Corp, Loveland, CO). Urine was centrifuged (500 x g for 5 min) and the supernatant was removed for urine protein and creatinine measurements (Vitros 250). The remaining supernatant was frozen within 4-6 hours of collection and stored at -80^{o}C. Before analysis, samples were thawed and divided into multiple aliquots so that multiple freeze-

thaw cycles could be avoided. Therefore, most samples underwent 2 freeze-thaw cycles before analysis.

Biomarker assays

Five proteins were measured in the urine from 20-25 dogs affected with XLHN and 10-19 normal age-matched littermates. In affected dogs, the proteins were measured every 2 weeks starting at the time point immediately preceding the development of proteinuria (UPC > 0.5) in each dog (generally 8-12 wks of age) until the onset of azotemia (sCr ≥ 1.2), after which they were measured monthly. This resulted in an average of 10 time points per dog (range 7-17). In the normal dogs, the proteins were measured at time points corresponding to their affected littermates for urinary NAG (uNAG) (average 10 time points per dog). However, for urinary RBP (uRBP), urinary B2M (uB2M), urinary NGAL (uNGAL), and uIgG they were measured at 4 time points, typically 2-3 months apart, due to the low levels of these proteins detected in their urine. Samples from each dog were run in duplicate fashion for each assay.

RBP, NGAL and IgG ELISA

uRBP, uNGAL, and uIgG were determined using either canine-specific (uNGAL: BioPorto Diagnostics A/S, Denmark; uIgG: Dog IgG ELISA Quantitation Kit, Bethyl Laboratories Inc., Montgomery, TX) or human-specific (uRBP: Immunology Consultants Laboratory Inc., Newberg, OR) sandwich ELISAs. All assays were commercially available and were used in accordance with the manufacturer's instructions. A commercially available quantitative assay for B2M that cross-reacts with

the canine protein was not available. For each immunoassay, the absorbance was measured at 450 nm using a microplate reader (BioTek Synergy 2, Winooski, VT) and the concentration of the urinary biomarker was interpolated from provided standards using a four-parameter logistic curve (Gen5 1.05, BioTek). Concentrations of analyte within each sample were then normalized to urine creatinine concentration and expressed as ratios (e.g. urinary RBP-to-creatinine ratio (uRBP/c)).

For the RBP assay, samples were diluted 1/5 to 1/4000 as necessary with provided diluent (phosphate buffered saline (PBS) containing bovine serum albumin, 0.25% Tween, and 0.1% Proclin300). 100 µl of either human RBP standards (7.8-250 ng/ml) or diluted sample was placed into duplicate wells that were pre-coated with affinity purified anti-Human RBP antibody (Ab). Samples were incubated for 1 hour, wells were washed four times with Wash solution (PBS containing 0.5% Tween), and 100 µl HRP-conjugated anti-human RBP Ab was added and incubated for 10 min in the dark. Wells were then washed four times and 100 µl substrate solution (3,3',5,5'-tetramethybenzidine (TMB) with hydrogen peroxide in citric acid buffer, pH 3.3) was added. The plate was incubated for 10 min in the dark, and the reaction was stopped with 100 µl stop solution (0.3 M sulfuric acid).

For the IgG assay, plates were first coated with 100 µl capture Ab (Sheep anti-Dog IgG-affinity purified Ab) diluted in coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6) and incubated for 1 hr. Three washes were performed (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) followed by blocking for 30 min with 200 µl Postcoat Solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0). After three washes, 100 µl of

each standard (7.8-500 ng/ml) and sample diluted 1/10 to 1/4000 as necessary with Diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) was added to duplicate wells and incubated for 1 hr. Wells were washed five times followed by addition of 100 µl HRP-labeled detection Ab (Sheep anti-Dog IgG-HRP conjugate, 1:40,000 dilution) and incubated for 1 hr. Wells were again washed five times, and 100 µl TMB substrate was added for 15 min. The reaction was stopped with 100 µl 2 M H_2SO_4 and the absorbance was determined after 5 min. All buffers used in this assay were reconstituted with mEq water, pH adjusted, and filtered (0.2 µm).

For the NGAL assay, samples were diluted 1/100 to 1/4000 as necessary with the diluent provided. Wells precoated with mouse monoclonal anti-dog NGAL Ab were incubated for 1 hr on a shaking platform with 100 µl standard (0-400 pg/ml) or diluted sample in duplicate wells. After three 1-min washes, 100 µl biotinylated mouse monoclonal anti-dog NGAL Ab was added for 1 hr on a shaking platform followed by three 1-min washes. 100 µl HRP-conjugated streptavidin was added and plates were incubated for 1 hr on a shaking platform, followed by three 1-min washes. 100 µl TMB substrate was added and the plate was incubated for 10 min in the dark. The reaction was stopped with sulfuric acid, and the absorbance was measured after 5 min at 450 nm using 620 nm as a reference wavelength.

NAG colorimetric assay

uNAG was evaluated using an automated enzymatic colorimetric assay (Diazyme Laboratories, Poway, CA) on a Roche Hitachi 911 analyzer (GMI Inc, Anoka, MN). In this assay, NAG hydrolyzes 2-methoxy-4-(2'nitrovinyl)-phenyl 2-acetamido-2-deoxy-β-

D-glucopyranoside (MNP-GlcNAc) to 2-methoxy-4-(2'-nitrovinyl)-phenol and the product formation is detected by measuring the absorbance at 505 nm after addition of an alkaline buffer. The assay was run in accordance with the manufacturer's instructions, except that all sample volumes were doubled to 20 µl instead of the 10 µl recommended. Results were divided by 2 to obtain final values. Calibration was performed before each run, and both provided controls and internal quality control samples were assayed for each sample run.

Analytical validation of IgG, NAG, RBP, and NGAL assays

To analytically validate the selected IgG and NAG assays for canine urine, and to ensure adequate assay performance in our laboratory for the RBP and NGAL assays, intra- and inter-assay variability, linearity, and spiking recovery were determined for the RBP, IgG, and NAG assays, and all but intra-assay variability was determined for the NGAL assay. In addition, assay sensitivity was determined for uNAG. For intra- and inter-assay variability, samples with low, middle, and high concentrations of analyte were assayed with 6-10 repetitions within one assay run or in consecutive assay runs. For each sample the coefficient of variation (CV) was calculated. To investigate linearity using dilutional parallelism, 4-5 dilutions were made for each sample (typically low, middle, and high concentrations), and observed to expected ratios were calculated. Spiking recovery was performed by adding a known amount of provided protein standard to samples with known concentrations for the RBP, IgG, and NAG assays. In addition, three canine urine samples of low, middle, and high concentrations were combined in various combinations for RBP, NGAL, and NAG assays. Observed to

expected ratios were calculated for each spiking experiment. Assay sensitivity for uNAG was calculated using 10 blank determinations of water. The detection limit was calculated using mean blank value + 2.6 x standard deviation,[154] and values below this were considered to be zero.

Influence of the number of freeze-thaw cycles was evaluated for uNGAL and uNAG on samples with low, middle, and high concentrations or activities by evaluating the same sample stored at -80^{o}C on different days. In addition, for uNAG, stability was evaluated for storage at room temperature (RT) for 2, 4, 8, 12, and 24 hrs and at 4^{o}C, -20^{o}C, and -80^{o}C for 2, 4, and 8 weeks for samples with low, middle and high activity. Stability was determined using linear regression.

Measurement of B2M by semi-quantitative Western blot

uB2M was evaluated by Western blot. Urine samples were normalized to 20 mg/dl creatinine and loaded on duplicate 15% SDS-polyacrylamide gels (Ready Gel Tris-HCl Gel, BioRad, Hercules, CA). Purified human B2M (0.05 μg/lane, Sigma, St. Louis, MO) was loaded on each gel in order to serve as a positive control as well as to allow for semi-quantitative assessment of uB2M in each urine sample. Protein was transferred to a nitrocellulose membrane, and membranes were blocked for 2 hrs with a 6% milk solution, incubated for 1 hr at room temperature with a polyclonal rabbit anti-human B2M antibody (1:4,000, Dako, Carpinteria, CA), washed for 15 min, and incubated for 1 hr with HRP-labeled secondary antibody (goat anti-rabbit, 1:10,000, Dako). The signal was detected using an enhanced chemiluminescent substrate (Pierce ECL Western Blotting Substrate, Thermo Scientific, Rockford, IL). Films were scanned

(Epson scanner) and integrated density for each band was quantified using ImageJ software.[141] The integrated density for each urine sample was divided by the integrated density from the standard control lane to obtain a semi-quantitative, unitless value for uB2M.

Glomerular filtration rate (GFR)

For 22 affected dogs and 19 normal littermates, dynamic renal scintigraphy using clearance of ^{99m}Tc-diethylenetriaminepentaacetic acid (DTPA) was performed to estimate GFR at monthly intervals starting at 9 weeks of age as described previously.[155,156] Briefly, ^{99m}Tc-DTPA was injected through a cephalic vein, and dogs were imaged using a large-field-of-view γ camera over 12 minutes. Regions of interest were drawn around each kidney, and the background was subtracted. Global GFR was estimated. Because GFR was determined much less frequently than other clinical data, GFR values for time points in between measurements were calculated by interpolation using the two bounding measurements. This provided more GFR values for correlation with other measures of renal function.

Histologic evaluation

Serial renal biopsies obtained from 10 affected dogs and 10 normal littermates were analyzed to provide information about structural changes in the glomeruli and tubulointerstitium over the course of disease, and the timing of these biopsies was standardized to allow comparisons between dogs in addition to serial comparisons within each dog. Initially, dogs were biopsied at time points defined by the dog's age (months

4, 6, 8, and 10). Later, dogs were biopsied when they reached specified "milestones" in the course of their disease (independent of age) that defined specific stages of disease progression: onset of persistent microalbuminuria, onset of a sCr ≥ 1.2 mg/dL (azotemia), onset of sCr ≥ 2.4 mg/dL, and onset of sCr ≥ 5 mg/dL (endpoint). Biopsies were obtained and processed as described previously.[155] Briefly, cores were immediately placed into 10% formalin and embedded in paraffin within 24 hrs. Three μm sections were cut from two levels of each biopsy 100 μm apart in order to represent a different plane of the biopsy. Sections stained with H&E, Masson's trichrome, and PAS were evaluated by a board-certified pathologist.

For scoring of the glomeruli, three serial sections were aligned, and only intact glomeruli were evaluated (i.e., tufts that were at the edge of the biopsy whose Bowman's capsules were disrupted were not scored). The percentage of glomeruli with the following features were assessed: normal (i.e., had none of the following lesions); segmental mesangial expansion; global mesangial expansion; synechia; fibrin, proteinaceous fluid and/or cells within Bowman's space (fibrinous crescent); obsolescence; and dilation of Bowman's capsule.

To score the tubulointerstitium, twenty randomly chosen 200X fields of tubulointerstitium were evaluated in two PAS and two H&E biopsy sections (5 fields per biopsy). Only the renal cortex was scored (scoring method outlined in Table 7). The scores (0-3) were added together so that the total score for each biopsy time point within each category ranged from 0 to 60. Additionally, trichrome sections were used to estimate interstitial fibrosis using Imagescope (Aperio Technologies, Inc, Vista, CA) to

outline each specimen as well as the regions of fibrosis. The total area of fibrosis was divided by the total area encompassed by the biopsy.

Table 7. Criteria for scoring lesions in the cortical tubulointerstitium based on evaluation of PAS and H&E biopsy sections.

Lesion	Scores for each group of lesions (each type of lesion in each group is scored separately)
• Tubular dilation • Presence of intact tubular membrane brush border • Tubular atrophy • Tubular cell vacuolation	0=lesion not present 1=lesion present in <50% tubules 2=lesion present in >50% tubules 3=lesion present in all tubules within the field
• Tubular single cell necrosis • Intratubular casts • Mineralization of tubular basement membranes	0=not present 1=present in 1 tubule in a 20x field 2=present in >5 tubules in a 20x field 3=present in >10 tubules in a 20x field
• Interstitial fibrosis	0=not present 1=mild focal fibrosis 2=moderate focal fibrosis 3=diffuse fibrosis
• Acute inflammation • Chronic inflammation	0=not present 1=inflammatory cells scattered throughout specimen, not aggregated 2=focal aggregate of inflammatory cells (>20 cells) 3=multiple large aggregates of inflammatory cells

Statistical methods

The effect of storage time on urinary biomarker determinations was evaluated with linear regression, whereby the mean values for each year and the overlap of their standard deviations were compared. Descriptive statistics for the clinical data for normal and affected dogs was calculated using medians and interquartile ranges. Comparison of

median values between normal and affected dogs at the time when sCr was ≤ 0.7 mg/dl was performed using nonparametric equality of medians test. Correlations between the urinary biomarkers and clinical and histologic variables were determined using Pearson's correlation coefficient with clustered robust standard errors. Significance was set at a p-value < 0.05. All statistical calculations were performed using Stata 11.0 (Stata Corp LP, College Station, TX).

Results

Analytical validation of IgG, NAG, RBP, and NGAL assays

Analytical validation results for each urinary biomarker are presented in Table 8. Mean intra- and inter-assay variability was acceptable for all assays (<10% and 15%, respectively) based both on standard criteria as well as ½ CV of the normal dogs.[129] However, for the NAG assay, intra- and inter-assay variability for the low activity samples (< 5 U/L) was higher (14% and 16%, respectively) than for the middle and high activity samples. Intra-assay variability was not evaluated for the NGAL assay for several reasons, including the high cost of the assay, the manufacturer-reported intra-assay variation of 1-2% for canine urine, and the low duplicate CV for samples in this study (typically 1-3%). Linearity for all assays was acceptable (Table 8), although it is important to note that this was true for the RBP assay only when the values obtained fell within the linear part of the standard curve (approximately 7.8-130 ng/ml); when above this value, concentrations were underestimated by 30-50%. Spiking recovery results

were acceptable for the IgG, RBP, and NGAL assays. For the NAG assay, spiking recovery was only acceptable when samples were spiked with NAG control or when samples with moderate to high activity were mixed together. However, when a sample with uNAG activity < 5 U/L was mixed with a sample with higher activity, the measured activity was much lower than expected (Table 8).

Table 8. Analytical validation results for quantitative assays for urinary biomarkers using canine urine.

	IgG	RBP	NAG	NGAL
Intra-assay CV (%)[a]	**9.6**	**4.8**	**7.1**	ND
Low	11.7	4.3	13.9	…
Middle	7.9	3.8	6.3	…
High	9.3	6.2	1.2	…
Inter-assay CV (%)[a]	**4.5**	**10.3**	**7.6**	**7.3**
Low	5.6	9.8	15.6	10.8
Middle	5.0	10.8	4.9	4.6
High	3.0	10.2	2.4	6.5
Linearity (%)	92-122	97-111	93-119	96-107
Spiking recovery (%)	86-117	70-116	96-111[b] 29-62[c]	90-101
Detection limit	ND	ND	0.4 U/L	ND

[a]Mean intra- and inter-assay CV's for all samples evaluated.
[b]Observed/expected % when using samples with moderate to high activity (>8 U/L)
[c]Observed/expected % when using a sample with low activity (<5 U/L) and a sample with moderate to high activity
CV, coefficient of variation; ND, Not determined.

Stability was evaluated for uNAG, and activity mildly but progressively increased at 8, 12 and 24 hrs at room temperature. Except for one sample that demonstrated a minimal but significant increase with storage at 4^{o}C, there was otherwise no significant change observed during storage for 2 months at 4^{o}C, -20^{o}C, or -80^{o}C, and

no significant difference was observed in each sample stored at different temperatures. The number of freeze-thaw cycles did not significantly alter measured uNAG activity for samples with moderate to high activity (up to 5 freeze-thaw cycles); however, a small but significant ($p = 0.025$) increase was observed for the sample with low uNAG activity (< 5 U/L). For uNGAL, the concentration was not significantly affected with up to 4 freeze-thaw cycles (Appendix A).

Because urine samples were stored for variable lengths of time before analysis, the effect of storage time on assay results was evaluated. No apparent storage effect was observed for uNAG, uRBP, uB2M, and uIgG. However, for uNGAL, samples evaluated after only two years of storage had consistently higher concentrations than samples evaluated after storage for four years or longer. However, further decreases in concentration for samples stored > 4 years was not evident (Appendix B).

Comparison of results for urinary biomarkers with clinical data

uIgG/c, uRBP/c and uB2M were low to absent in the normal males (Table 9). However, uNGAL/c in normal puppies <4 months of age was frequently substantially higher than in older dogs (although still generally lower than most measurements in the affected males). uNAG/c was generally low in the normal males; however, variable increases were seen relatively frequently at a young age and occasionally at older ages as well. This resulted in some overlap of uNAG/c in normal males and affected males (Table 9). For both uNGAL/c and uNAG/c, these occasional high results in the normal dogs resulted in higher means as compared with median values. For uB2M, a faint band

was observed in at least one urine sample from 4 of the 18 normal male dogs evaluated by Western blot.

**Note that for each of the following tables and graphs, abbreviations are defined as follows: uRBP/c, urine retinol binding protein:creatinine ratio; uIgG/c, urine immunoglobulin G:creatinine ratio; uNAG/c, urine N-acetyl-β-D-glucosaminidase:creatinine ratio; uB2M, urinary β2-microglobulin; uNGAL/c, urine neutrophil gelatinase-associated lipocalin:creatinine ratio.

Table 9. Group descriptive statistics (median, range) for clinical data from all normal dogs and for normal and XLHN dogs when sCr ≤ 0.7 mg/dl.

	Normal dogs (all ages)		**Normal dogs when sCr ≤ 0.7 mg/dl**		**XLHN dogs when sCr ≤ 0.7 mg/dl**	
	N	**Median (IQR)**	**N**	**Median (IQR)**	**N**	**Median (IQR)**
sCr (mg/dl)	376	0.8 (0.3)	162	0.6 (0.2)	196	0.6 (0.1)
GFR (ml/min/kg)	317	3.17 (0.58)	116	3.38 (0.68)	105	3.87 (0.96)[a]
UPC	310	0.20 (0.24)	117	0.26 (0.28)	160	0.60 (3.0)[a]
uRBP/c (mg/g)	32	0.08 (0.08)	11	0.15 (0.07)	86	0.38 (2.4)[c]
uB2M	69	0.00 (0)	24	0.00 (0)	98	0.01 (0.29)[b]
uNAG/c (U/g)	185	0.4 (3.8)	71	1.9 (8.4)	81	8.1 (15.6)[a]
uNGAL/c (µg/g)	48	1.6 (5.2)	16	7.9 (14.6)	79	34.6 (99.6)[d]
uIgG/c (mg/g)	57	2.2 (1.4)	19	3.0 (1.8)	98	48.0 (281)[a]

[a]P < 0.001, [b]P = 0.001, [c]P = 0.002, and [d]P = 0.003 compared with normal dogs when sCr < 0.8 mg/dl; P value for sCr = 0.066

sCr, serum creatinine; GFR, glomerular filtration rate; UPC, urine protein:creatinine ratio; N, number of observations; IQR, interquartile range (i.e., difference between the third and first quartiles).

In the affected dogs, each urinary biomarker was significantly increased early in the disease process (i.e., when sCr was not yet elevated) as compared with normal dogs

(Table 9). uIgG/c, uNAG/c, uNGAL/c, and uB2M all demonstrated an increase during early stages of the disease, and all but uNAG/c appeared to continue to increase during mid- to late-stage renal disease progression (as defined by sCr concentration) based on median values. However, comparison of the ranges for these biomarkers at each interval showed substantial overlap, indicating that they remained relatively constant in later stages (Figure 10). In contrast, uRBP/c showed a progressive increase at all intervals, most pronounced in the mid- to late-stages of renal disease progression. Although not evident from the figure, median uRBP/c is significantly higher (3 mg/g; IQR=10 mg/g) when sCr is 0.6-1.2 mg/dl as compared to when sCr < 0.6 mg/dl (0.26 mg/g; IQR=0.9 mg/g).

Each urinary biomarker was also plotted against defined intervals of GFR and UPC (Figures 11 and 12). For GFR, results are similar to sCr with each biomarker increasing until a time point of moderate impairment of renal function (GFR 1.5-2.5 ml/min/kg), although overlap of the interquartile range was substantial for all biomarkers except uRPB/c. However, at a time point of end-stage renal impairment (GFR <1.5 ml/min/kg) median values for all biomarkers stayed at a similar level or decreased slightly, except for uRBP/c, which continued to show a marked increase.

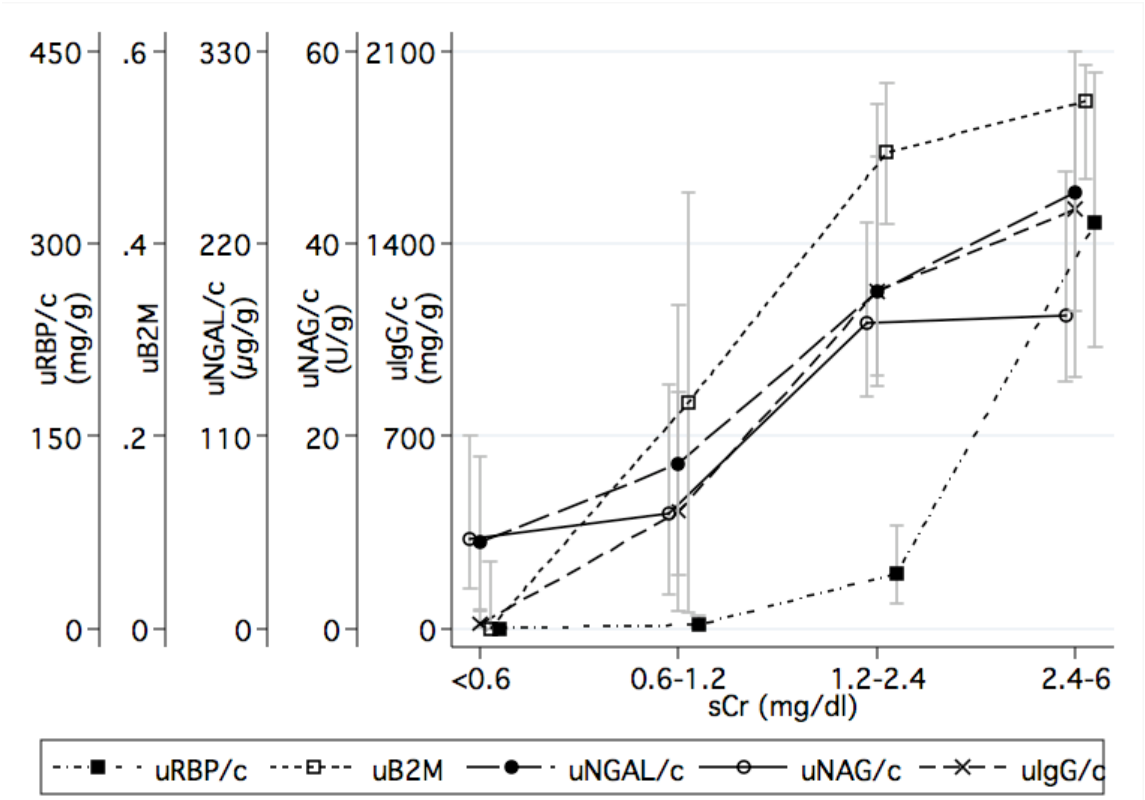

Figure 10. Plot of the median values for five urinary biomarkers for 4 different disease stages as defined by serum creatinine (sCr) concentrations in dogs with XLHN. All biomarkers show a progressive increase until mid-azotemia (sCr 1.2-2.4 mg/dl). However, only uRBP/c continues to demonstrate a progressive increase until end-stage disease. Markers indicate median values and the bars indicate the interquartile range.

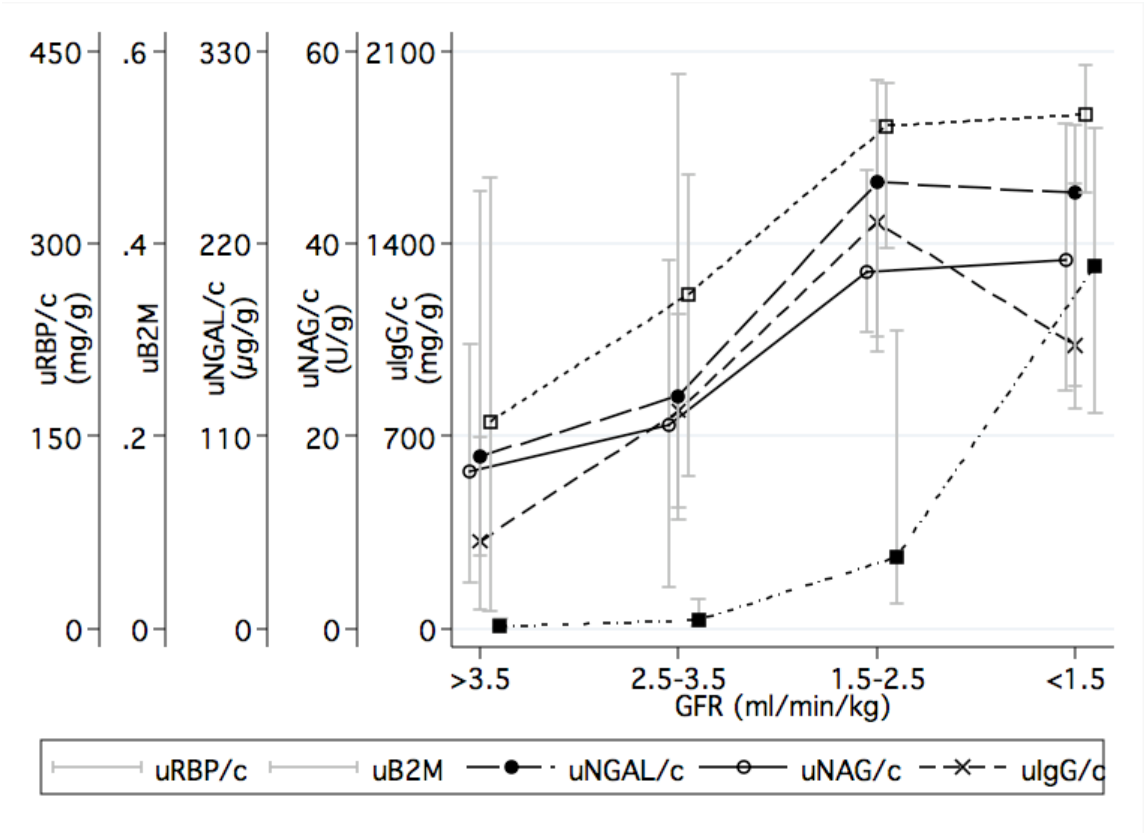

Figure 11. Plot of the median values for five urinary biomarkers for 4 different disease stages as defined by glomerular filtration rate (GFR) in dogs with XLHN. All biomarkers show a progressive increase until evidence for a moderate decline of renal function (GFR < 2.5). Only uRBP/c continues to increase with end-stage renal impairment. Markers indicate median values and the bars indicate the interquartile range.

As compared with UPC (Figure 12), uNGAL/c, uNAG/c, and uIgG/c were all significantly higher than in the normal dogs when UPC < 0.5. Most of the urinary biomarkers demonstrate a substantial increase only when UPC > 2; however, median uIgG/c and uNGAL/c increased throughout all UPC intervals. uRBP/c, uB2M, and uNAG/c remained relatively stable until UPC > 2, although the increase in median uRBP/c was much less pronounced than for the other biomarkers. Particularly marked increases were observed in all biomarkers when UPC > 10. It should be noted that

median GFR and sCr concentration were 3.5 ml/min/kg and 0.8 mg/dl, respectively, for the UPC interval of 2-10 as compared with 2.2 ml/min/kg and 1.8 mg/dl, respectively, when the UPC was >10.

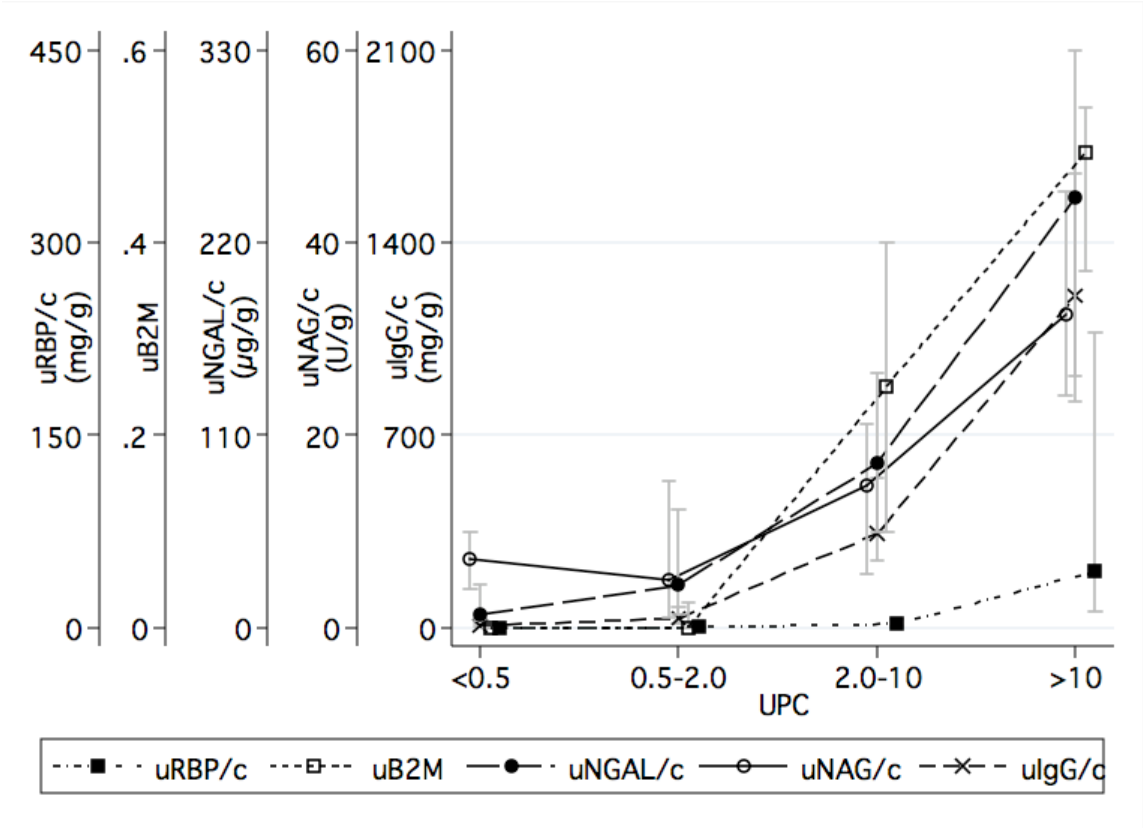

Figure 12. Plot of the median values for five urinary biomarkers for 4 different disease stages as defined by urine protein:creatinine ratio (UPC) intervals in dogs with XLHN. Most urinary biomarkers demonstrate a substantial increase only after UPC > 2. Markers indicate median values and the bars indicate the interquartile range.

Correlations of each urinary biomarker with sCr, GFR, and UPC are presented in Figure 13. Serum creatinine correlated most strongly with GFR and uRBP/c, with a moderate correlation observed with UPC. Similarly, GFR correlated most strongly with sCr and had a moderate correlation with uRBP/c and UPC. All biomarkers showed a

moderate to high correlation with the magnitude of proteinuria. UPC correlated most strongly with uIgG/c with the least correlation being observed with uRBP/c. Urine specific gravity showed poor correlation (< 0.4) with clinical data and urinary biomarkers (Appendix C).

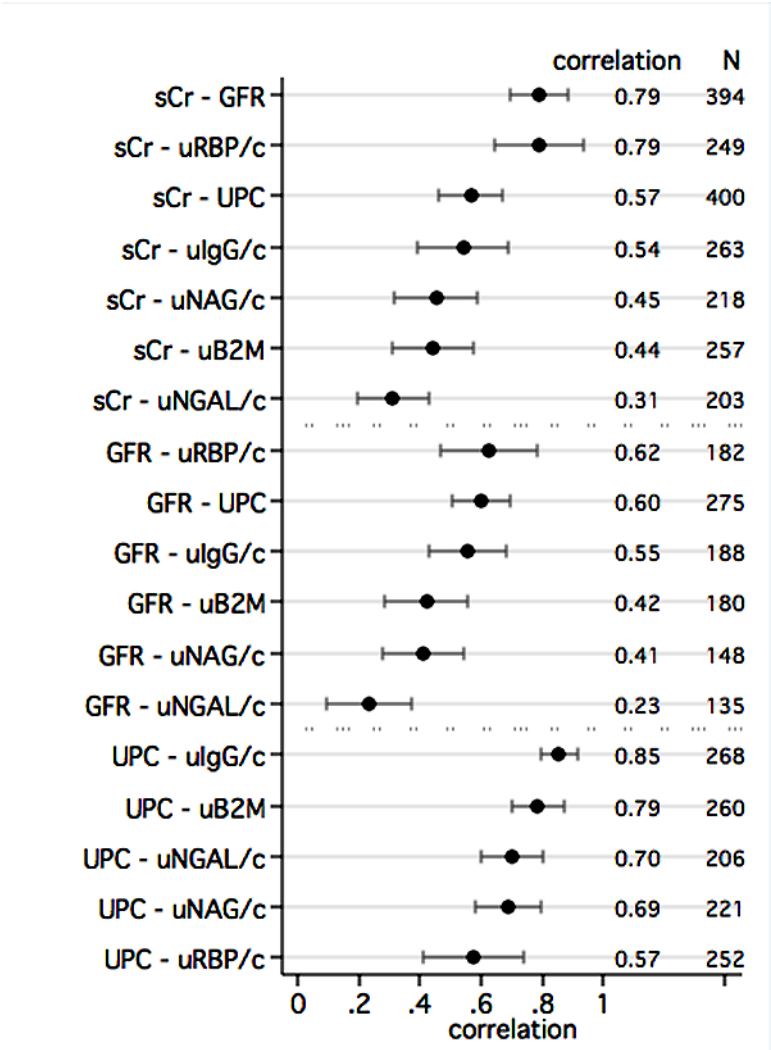

Figure 13. Correlation of standard clinical measures of renal function and urinary biomarkers in dogs with XLHN. Correlations with GFR are negative. Circles indicate the estimated correlation and bars indicate its confidence interval. N, number of paired observations; sCr, serum creatinine; GFR, glomerular filtration rate; UPC, urine protein:creatinine ratio.

Correlations of the urinary biomarkers with each other were also performed (Table 10). uB2M, uNGAL/c, and uIgG/c were all strongly correlated with one another and these also demonstrated moderate correlation with uNAG/c. Only uIgG/c was moderately correlated with uRBP/c.

Table 10. Correlations (Corr) of urinary biomarkers in dogs with XLHN. For all correlations $P < 0.001$

		uB2M	**uNGAL/c**	**uNAG/c**	**uIgG/c**
uRBP/c	Corr (n)	0.453 (247)	0.317 (195)	0.392 (207)	0.605 (252)
uB2M	Corr (n)	…..	0.768 (201)	0.600 (214)	0.696 (260)
uNGAL/c	Corr (n)	…..	…..	0.606 (181)	0.721 (205)
uNAG/c	Corr (n)	…..	…..	…..	0.607 (219)

n, number of paired observations.

Histologic analysis

Mean histology scores were compared between affected and normal dogs, and correlations of glomerular and tubulointerstitial lesions were performed with one another using combined data from both normal and affected dogs, affected dogs alone, and normal dogs alone (Appendix C). All glomerular and tubulointerstitial categories were significantly different in the affected dogs as compared with their normal littermates. In addition, significant correlations between most categories were present when data points from all dogs were evaluated together. These findings indicate that the presence of lesions distinguishes affected dogs from normal dogs. However, approximately 10-15% of the biopsies from normal dogs demonstrated the appearance of segmental mesangial expansion. In addition, in normal dogs, the tubules frequently contained 1-2 small

vacuoles and rare tubular dilation was observed. When only affected dogs were evaluated, the majority of tubular lesions were positively correlated with more severe glomerular damage (i.e., global mesangial expansion, obsolescence, and synechia). The majority of tubular lesions also correlated strongly with each other except for tubular cell vacuolation, tubular basement membrane mineralization, and tubulointerstitial acute inflammation. In particular, fibrosis scored using random fields on H&E and PAS stained slides correlated very strongly with the overall percentage of fibrosis estimated from the trichrome stained slide ($r=0.928$). Therefore, these two categories were combined for the remaining correlations.

When comparing histologic findings with conventional tests of renal function in all dogs, the presence of histologic lesions correlated with a decreasing GFR and an increasing sCr and UPC. However, urine specific gravity showed poor correlation with biopsy findings. When correlations were performed in affected dogs only (Table 11), GFR and sCr demonstrated moderate to strong correlation with most tubular categories, and correlations were particularly strong for the degree of interstitial fibrosis. This was typically true for the UPC as well. Overall, correlations with clinical data were stronger for tubulointerstitial lesions than for glomerular lesions. However, a decreasing GFR and increasing sCr also strongly correlated with the presence of more severe glomerular lesions. In the normal dogs, no significant correlations were present with any histologic category in relation to clinical data or urinary biomarkers (Appendix C). Segmental mesangial expansion, fibrinous crescents, tubular cell vacuolation, tubular basement

membrane mineralization, and tubulointerstitial acute inflammation did not correlate with any clinical findings (Appendix C).

Table 11. Correlation of histologic lesions with conventional measures of renal function in dogs with XLHN.

Glomerular lesions	GFR	sCr	UPC
Normal & mild	0.748**	-0.622*	-0.717**
Moderate & severe	-0.829**	0.744**	0.527
Synechia	-0.571	0.518	0.526
Fibrinous crescents	-0.165	0.120	0.491
Bowman's capsule dilation	-0.431	0.395	0.600
Tubulointerstitial lesions			
Interstitial fibrosis	-0.835**	0.810**	0.691*
Chronic interstitial inflammation	-0.798**	0.625	0.761**
Tubular single cell necrosis	-0.753**	0.670*	0.710*
Tubular atrophy	-0.746**	0.675*	0.523
Tubular dilation	-0.753**	0.705*	0.669*
Tubular casts	-0.712*	0.706*	0.387
Lack of brush border	-0.782**	0.723*	0.699*

*P < 0.05; **P < 0.001
Normal & mild glomerular lesions: normal + segmental mesangial expansion; Moderate & severe glomerular lesions: global mesangial expansion + obsolescence. GFR, glomerular filtration rate; sCr, serum creatinine; UPC, urine protein:creatinine ratio.

When comparing histologic findings with the urinary biomarkers in all dogs, significant correlations were observed for nearly every category for each biomarker except for uRBP/c, which was positively correlated only with more severe glomerular and tubular lesions as well as the presence of an intact brush border (Appendix C). When only data from affected dogs was used in the calculations (Table 12), a significant positive correlation was observed between uRBP/c with more advanced tubulointerstitial

lesions (i.e., interstitial fibrosis) as well as with global mesangial expansion (Appendix C). uB2M correlated with the majority of glomerular and tubulointerstitial categories. Interestingly, UPC and uIgG/c negatively correlated with the proportion of normal or mildly affected glomeruli, but they did not correlate with severity of the glomerular lesion. Both uIgG/c and uNGAL/c demonstrated strong positive correlation with the proportion of glomeruli with dilated Bowman's capsule. uNAG/c did not significantly correlate with any glomerular lesions, and uNAG/c, uNGAL/c, and uIgG/c did not significantly correlate with any tubulointerstitial lesions.

Table 12. Correlation of histologic lesions with urinary biomarkers in dogs with XLHN.

Glomerular lesions	**uRBP/c**	**uB2M**	**uNGAL/c**	**uNAG/c**	**uIgG/c**
Normal & mild	-0.494	-0.846**	-0.660	-0.537	-0.618*
Moderate & severe	0.610	0.630*	0.195	0.449	0.331
Synechia	0.292	0.627*	0.172	0.525	0.303
Fibrinous crescents	0.239	0.591	0.554	0.385	0.337
Bowman's capsule dilation	0.346	0.666*	0.858**	0.534	0.743**
Tubulointerstitial lesions					
Interstitial fibrosis	0.692*	0.692*	0.425	0.540	0.621
Chronic interstitial inflammation	0.456	0.790**	0.420	0.603	0.589
Tubular single cell necrosis	0.416	0.838**	0.521	0.607	0.547
Tubular atrophy	0.414	0.655*	0.287	0.490	0.460
Tubular dilation	0.713*	0.656*	0.431	0.489	0.605
Tubular casts	0.369	0.456	0.044	0.409	0.216
Lack of brush border	0.522	0.708*	0.447	0.430	0.625

*P < 0.05; **P < 0.001

Normal & mild glomerular lesions: normal + segmental mesangial expansion; Moderate & severe glomerular lesions: global mesangial expansion + obsolescence.

Discussion

The purpose of this study was to serially assay a panel of urinary biomarkers in a canine model of naturally occurring progressive glomerular disease and to correlate these biomarkers with standard measures of tubular and glomerular function and histopathologically assessed damage in order to assess their value for detecting the onset and gauging the progression of renal disease. In this study, dogs affected with XLHN demonstrated marked increases in all biomarkers, often very early in their disease process, whereas normal littermates had little to no urinary biomarkers detected. A progressive increase was observed for uRBP/c, whereas for all other biomarkers, an initial increase followed by a relatively constant protein excretion was detected. uRBP/c correlated best with sCr and GFR, and it also showed a significant positive correlation with interstitial fibrosis. Results of the present study also indicate acceptable assay performance in canine urine using ELISAs for RBP, NGAL, and IgG, and an enzymatic colorimetric assay for NAG.

The results of this study provide new information regarding urinary biomarkers in dogs with CKD. uNAG and uNGAL have not previously been serially evaluated in dogs with CKD, and this is the first report measuring uNGAL in dogs. uRBP, uB2M, and uIgG have been serially evaluated in the urine from dogs with XLHN using semi-quantitative methods.[104] However, the present study provides a quantitative analysis for both uRBP/c and uIgG/c, and more dogs and time points were used for the analysis of the proteins in this study. While uB2M was not quantitatively measured, trends are likely

valid based on the results for uRBP, where a strong correlation between the Western blot (see Chapter III) and the ELISA results was observed ($r = 0.871$).

Analytical assay validation procedures were performed on all quantitative assays used in order to demonstrate acceptable analytical assay performance using canine urine in our laboratory. Validation results for several of these assays have previously been reported.[103,114] In the present study, similar precision and linearity results were obtained using the same assay for uRBP and different assays for uIgG and uNAG as compared with previous studies (Table 8).[103,114] In addition, detection limits for uNAG activity were similar. However, additional accuracy evaluation was performed in the present study using spiking recovery. Poor recovery was demonstrated for uNAG activity when a sample with low activity was added to one with high activity. This finding may be explained by the presence of an inhibitor of NAG activity in urine samples.[157] The present study also revealed that caution must be used when interpreting results for uRBP that fall outside the linear part of the standard curve. Analytical validation results for canine urine samples using the NGAL assay are available from the manufacturer, and results from the present study support the good performance of this assay. Overall, results from this study indicate that assays developed specifically for measurement of the canine protein performed well (i.e., NGAL, IgG), whereas those developed originally for other species (i.e., RBP, NAG) performed reasonably well but had a few caveats that need to be addressed when determining sample concentration.

NAG activity appears to be relatively stable in canine urine, with no significant effect seen with storage at $4^{o}C$, $-20^{o}C$, $-80^{o}C$ for up to 2 months, similar to previous

findings in dogs and people.[157,158] However, uNAG activity increased with storage at room temperature, indicating the need to refrigerate urine samples as soon as possible after collection. Surprisingly, uNAG in the low-activity sample increased with freeze-thaw cycles in the present study, whereas uNAG activity in cats significantly decreased, but only after the 5th freeze-thaw cycle.[112] This difference may be due to methodology or species differences. uNAG activity in samples with moderate to high activity levels and uNGAL concentration appears to be stable over multiple freeze-thaw cycles.

In normal dogs, urinary biomarkers were minimal to absent (Table 9), consistent with previous studies,[103,104,114,159] and the quantitative values obtained in the present study are similar to those reported for uNAG/c[159] and uRBP/c.[103] uNGAL/c was occasionally substantially higher in puppies <4 months of age than in older dogs. This finding could be due to contamination of the urine with preputial leukocytes due to difficulty in obtaining a clean mid-stream urine sample from puppies at this age. Indeed, on urine sediment analysis, it was common to see mild to moderate pyuria until approximately 4 months of age. For NAG, variable increases were seen not only at a young age, but occasionally at older ages as well. These transient increases resulted in uNAG/c similar to those observed during early stages of renal disease in the affected dogs, and they may be a result of semen or leukocyte contamination[157,160,161] or possibly of transient tubular damage.

All of the urinary biomarkers were higher in the dogs affected with XLHN than in their age-matched littermates very early in their disease process (Table 9). As compared with sCr and GFR, uRBP/c was the only urinary biomarker that showed a

clearly progressive increase with declining renal function (Figures 10 and 11). This urinary marker was also least influenced by proteinuria and showed a moderate to strong positive correlation with interstitial fibrosis (Table 12). uB2M also correlated strongly with interstitial fibrosis; however, it also correlated with many other tubular lesions, which may indicate a lack of specificity for irreversible tubulointerstitial damage. uB2M, uNAG/c, uNGAL/c, and uIgG/c all increased until a stage of moderate azotemia, but they remained relatively stable afterwards, and when GFR < 1.5 ml/min/kg, median uNGAL/c and uIgG/c actually decreased (Figure 11). This phenomenon has been seen with UPC in end-stage renal failure and indicates that one should not use a decrease in urinary protein excretion as a sign of improvement in renal disease if the disease is otherwise worsening.[12]

Figure 12 demonstrates that proteinuria occurs well before a detectable decline in renal function in these dogs, and that proteinuria may be influencing the presence of many of the markers, particularly early during the disease process. For example, when compared with UPC, all proteins demonstrated the greatest increase when UPC > 2, and especially large increases were seen when UPC > 10. It is important to note that these dogs exhibited marked non-selective proteinuria at an early age, and this combined with the large magnitude of proteinuria typical for their disease illustrates one of the main limitations in evaluating urinary biomarkers in patients with proteinuric renal disease. Namely, increased filtration of intermediate and high molecular weight proteins can interfere with reabsorption of low molecular weight proteins (LMW) by the renal tubules. Therefore, the relative contribution of overt proteinuria as opposed to tubular

injury and declining tubular function on the increased presence of low molecular weight proteins is difficult to differentiate early on. However, the observation of normal uRBP in human patients with marked, but selective proteinuria suggests that competition for reabsorption may not be a major factor for the presence of RBP in urine.[84] uNAG can increase due to increased lysosomal turnover, secondary to proteinuria, in the absence of detectable tubular damage.[95] Later in the disease, there is substantial evidence in these dogs to support tubular damage and impaired tubular function based on histology, decreasing GFR, and increasing sCr. While the initial increases in the proteins evaluated may indicate early tubular disease, further studies are needed to determine the contribution of proteinuria alone from tubular damage and dysfunction.

The urinary biomarkers in this study were correlated with standard measures of renal function and damage, and these correlations were used to help determine which biomarkers might be most useful to include in a panel for renal disease detection and monitoring. Of the urinary biomarkers, uRBP/c had the strongest correlation with sCr and was also highly correlated with a declining GFR and increasing degree of interstitial fibrosis (Figure 13, Table 12). This may indicate that uRBP/c might be especially useful for detecting early renal disease before an obvious increase in sCr is noted, particularly given the wide range of normal sCr that is possible in different dogs. The progressive increase in uRBP/c may also support its use in monitoring the progression of renal disease to help distinguish between pre-renal and renal influences contributing to an increased sCr, although this area warrants further investigation. Importantly, uRBP/c had the lowest correlation with UPC, which supports its use for detecting onset and

progression of renal disease in proteinuric patients since its increase may be more likely to be secondary to tubular dysfunction rather than simply competition from glomerular proteinuria.

uB2M showed a relatively poor correlation with sCr and GFR (Figure 13) and therefore, monitoring of uB2M does not appear to be helpful in mid- to late-stage renal failure. In addition, because of its excellent correlation with UPC, its use for early detection of clinically significantly decreased tubular function is questionable in proteinuric CKD. The marked and early increase in uB2M may indicate a low threshold for reabsorption that is easily overwhelmed by competition from intermediate and high molecular weight proteins or very mild tubular dysfunction.

In contrast to uB2M, the continual increase in uRBP/c suggests more effort by the tubules to conserve this protein when possible. This finding is in contrast to that found in a study in people, where RBP uptake by the tubules was saturated at the same stage of renal insufficiency as was that for B2M.[87] The reason for this difference may be the types of patients evaluated in this study (primary tubular disease), where competition for reabsorption by abnormally filtered proteins was not a factor. The tubular receptor, megalin, has a high affinity for RBP and vitamin D-binding protein, which was also found to show an early and progressive increase in these dogs.[104] It is interesting to speculate that megalin has a high affinity for these proteins because of their binding to important biological molecules, and that these types of proteins that are excreted in the urine would provide a more useful indication of tubular dysfunction in patients with protein-losing nephropathies than proteins without a particular need for conservation.

For those urinary biomarkers whose appearance in the urine was due to tubular damage (uNAG/c and uNGAL/c), poor correlation with GFR and sCr was seen; however, strong positive correlation with UPC was observed (Figure 13). These findings correspond with studies in cats showing that uNAG/c correlated with proteinuria but did not correlate with serum creatinine concentration,[112] and it did not serve as an independent predictor of the development of azotemia if UPC was included in the statistical model.[113] NAG is a ~150 kDa lysosomal enzyme within the proximal renal tubules that is released with tubular injury, while NGAL is a 25 kDa protein whose production is upregulated in injured renal tubular cells. However, NGAL also circulates in blood. Both of these proteins have been shown to increase with tubular damage,[91,99] and in this context, the lack of correlation with renal function is not surprising if these proteins are indicating a constant level of tubular damage in these dogs. However, it is surprising that neither of these proteins correlated with any of the tubulointerstitial lesions observed on histology, and the utility of both of these proteins for detecting ongoing tubular damage in the absence of other influences is questionable, as suggested by the strong correlation with UPC. uNAG/c may be increased due to proteinuria and subsequent increased lysosomal turnover without evidence of tubular injury,[95] and in theory it might also increase secondary to glomerular damage. However, several studies have shown that the contribution of glomerular damage negligibly affects uNAG activity in people.[92,93] In addition, uNAG/c showed only a moderate correlation with uIgG/c in the present study even though it is similar in size to IgG (Table 10). Isoenzyme evaluation would be necessary to further investigate glomerular versus tubular

contribution to uNAG activity in these dogs. Similarly, although NGAL expression is upregulated in the renal tubules of people with certain glomerular diseases, marked increases have also been observed in the urine of patients with marked proteinuria without evident tubular impairment based on GFR and sCr.[99] Because NGAL is a circulating LMW protein, decreased tubular reabsorption due to competition and worsening tubular function may be more responsible for its urinary increase in proteinuric CKD as opposed to tubular injury. The excellent correlation between uB2M and uNGAL/c would support this assertion (Table 10). However, it is likely that a combination of decreased reabsorption of NGAL by the tubules in addition to increased production by damaged tubules is occurring in proteinuric CKD.[99] Therefore, uNAG/c may be more strongly recommended as a marker of tubular injury than uNGAL/c.

uIgG/c demonstrated an excellent correlation with UPC (Figure 13), which is not surprising given that both measurements can provide an indication of the severity of glomerular compromise. This strong correlation supports that, at least in XLHN dogs, determination of uIgG/c does not provide any information beyond that obtained from the UPC. However, this would not be expected in patients with diseases where selective proteinuria occurs. For instance, in people marked proteinuria can be present in certain diseases without a compromise in the ability of the glomerulus to exclude HMW proteins (i.e., selective proteinuria).[29] Importantly, selective proteinuria corresponds with a favorable prognosis in these patients while a prognosis is worse in those cases of non-selective proteinuria, where passage of HMW proteins (e.g., IgG) occurs.[29,80] Therefore, despite the lack of unique value in this study, uIgG/c may provide useful information in

dogs with other causes of proteinuric renal disease, although selective proteinuria appears to be quite rare in dogs with proteinuric disease (personal observations).

One particular point of interest regarding uIgG/c is that a significant (although relatively small) increase was found in the earliest stages of disease in the affected dogs as compared to the normal dogs, often even before an increase in UPC. This finding of an increase in uIgG so early in these dogs may seem surprising given that uIgG is typically thought to indicate severe glomerular damage. However, a study in another dog model of X-linked hereditary nephropathy demonstrated focal ultrastructural basement membrane changes by 1 month of age.[162] Therefore, the increased uIgG/c in the current study is likely due to few and scattered basement membrane alterations that are enough to result in overall increased passage of HMW proteins without a concurrent increase in UPC. It is interesting to note, however, that both UPC and IgG negatively correlated with the proportion of normal or mildly affected glomeruli on histologic analysis, but they did not correlate with severity of the glomerular lesion (Tables 11 and 12). This implies that either these markers of glomerular damage are more influenced by the number of glomeruli affected rather than how severely an individual glomerulus is affected, or that severely affected glomeruli are nonfunctional and therefore do not contribute to proteinuria.

Verification of the utility (or lack thereof) of all of the urinary biomarkers evaluated in this study needs to be performed in a more diverse population of dogs with CKD, ideally in a serial fashion. However, based on the results of this study, uRBP/c is the only specific urinary protein that can be strongly recommended for monitoring

disease progression in male dogs with XLHN, and further evaluation of this protein is warranted in patients with progressive CKD due to a variety of causes. It could also be worthwhile to study other urinary markers in dogs with other causes of renal failure. In particular, uNAG/c and uIgG/c may still be important to include in a urinary biomarker panel, since these proteins appear to provide unique and sensitive information as compared with standard measures of renal function. It should be pointed out that this study showed a strong negative correlation between sCr and GFR (Figure 13). This finding was expected; however, sCr is more easily and less expensively measured than GFR. Therefore, when frequent serial measurements can be made, these results support the use of sCr to monitor renal disease.

One of the major limitations of this study was the variable storage time of the urine before analysis. While the results of this study support the short-term stability of uNAG and the relative resistance to freeze-thaw cycles for uNAG and uNGAL, the effects of long-term storage on all of these biomarkers is unknown. The length of time samples were frozen before analysis in this study ranged from 6 months to 8 years. However, no significant differences were observed when comparing values obtained in older samples to those obtained in newer samples, except for NGAL, where the more recent samples resulted in values anywhere from 10-100% higher than in older samples. Whether this represents dog-specific or storage-specific changes, however, is unknown. In addition, only 4-10 months separated the analysis time in samples from a single dog, which should minimize any adverse effect of variable length of sample storage on the trends observed. Lastly, the concentrations obtained for both normal and affected dogs

was similar to those reported by other studies in dogs with CKD where a comparison was possible. This supports that the proteins evaluated in this study are all relatively robust with regard to long-term storage at -80^{o}C, and that interpretations made from the analysis of these samples is likely to be minimally affected by storage time.

Another limitation of this study is that a single disease process was evaluated. While this allowed extensive serial evaluation of many variables, caution should be used before extrapolating results to dogs with glomerular disease in general. In addition, only male dogs were evaluated for this study. However, while this may result in slight differences in normal animals, there is no reason to suspect a difference in urinary proteomics between males and females during renal disease progression.

In summary, urinary excretion of tubular proteins in progressive renal disease differs depending on the protein, even when a similar mechanism for appearance of the protein in the urine is postulated. All of the urinary biomarkers evaluated increase early in the disease process in dogs with XLHN; however, only uRBP/c appears to be strongly correlated with renal disease progression. Based on these results, we believe that measurement of uRBP/c might be clinically useful for the early detection and monitoring of CKD in dogs, and a canine specific assay for uRBP would be helpful for this purpose. In addition, uNAG/c and uIgG/c may provide unique information in dogs with proteinuric CKD, although future studies are needed to determine the utility of these proteins beyond current measures of renal function. In addition, while this study provides a baseline for evaluating these proteins in dogs with CKD due to glomerular

disease, future studies are needed to determine if similar trends are seen with CKD due to a variety of other causes.

Ultimately, it might be reasonable to propose that a panel of urinary biomarkers will be able to more accurately predict the degree of renal function and chronic tubulointerstitial injury and fibrosis than is currently available with non-invasive methods, thereby helping clinicians to non-invasively determine appropriate treatment and prognosis in dogs with CKD. However, additional studies utilizing more renal biomarkers in both serum and urine are needed in order to identify a panel that can provide a more useful measure of renal injury and function during chronic renal disease in dogs.

CHAPTER V

CONCLUDING REMARKS

Characterization of proteinuria is currently one of the most promising areas for the early detection and monitoring of kidney disease in both human and veterinary patients. In this report, both quantitative and qualitative characteristics of proteinuria in dogs with kidney disease caused by X-linked hereditary nephropathy are presented. This family of dogs serves as a naturally occurring canine model of progressive proteinuric nephropathy, and the serial characterization of urinary proteins in this model will help provide the basis for future studies in dogs with chronic kidney disease due to other causes.

In order to aid clinicians in the monitoring of proteinuric renal disease, Chapter II describes the variability of the UPC in female dogs that are carriers for XLHN. The carrier female dogs were utilized for this study since they have documented stable proteinuria over long periods of time so that the day-to-day variability in the magnitude of proteinuria is not expected to change as a result of their disease process. Results showed that while the magnitude of proteinuria was reasonably similar over time within a single dog, a substantial degree of variability was present in measurements from one day as compared to the previous day. This indicates that clinicians should exercise caution before attributing a change in the UPC to treatment or disease progression, unless the value changes by at least 35% when UPC values are high (i.e., when the UPC nears 12), and up to 50-100% at lower UPC values (UPC < 3). Prior to this study,

variability of the UPC had not been analyzed in dogs. Hence, this study currently provides the only estimate of the day-to-day variability of the UPC in proteinuric dogs, and veterinarians in practice have meanwhile started to actively use the results of this study when monitoring treatment and progression of dogs with proteinuric renal disease.

It is clear that specific types of proteins can be as or more important than the magnitude of proteinuria in the diagnosis and monitoring of renal disease progression. In addition, some studies have found specific protein analysis to more accurately predict the outcome of renal disease than serum creatinine and histologic evaluation of renal biopsies in people. Therefore, the last two chapters qualitatively characterized proteinuria in male dogs affected with XLHN that serve as a model of progressive CKD due to glomerular disease.

In Chapter III, discovery proteomic techniques (SELDI and 2-D DIGE) were used to evaluate the urine proteome in the affected males at two relatively early time points during their disease process in order to target the identification of urinary biomarkers of early tubulointerstitial injury. This evaluation revealed a number of proteins that were differentially present in the urine between the two time points. Identification of established biomarkers of renal disease using both of these techniques helped validate their use for the discovery of urinary biomarkers in dogs. In addition, a number of proteins were identified that have not been previously implicated in canine renal disease. Only one other study has previously utilized a global proteomic technique for the evaluation of urine from dogs with CKD. The present report provides abundant

additional information that can help to plan future studies of the urine proteome and specific urinary protein evaluation in dogs with proteinuric kidney disease.

In Chapter IV, assays for specific proteins were used to either quantitatively or semi-quantitatively evaluate four urinary biomarkers associated with tubular injury or function and one marker associated with glomerular damage. These proteins were correlated with conventional tests of renal function and damage in order to characterize the timing of their appearance and their relative quantity during the progression of canine CKD. Results showed that all of the urinary biomarkers were increased in affected male dogs early during their disease process as compared with normal age-matched littermates. However, of the proteins evaluated, only retinol binding protein also correlated with later stages of disease progression. In addition, retinol binding protein appeared to be least influenced by proteinuria and therefore the most appropriate of the four biomarkers evaluated for monitoring tubulointerstitial disease progression secondary to glomerular disease. This study also analytically validated the various assays as well as tested the stability for two of the proteins. This data is currently lacking in the veterinary literature and will help future investigators better utilize and evaluate these assays and proteins.

This investigation is the first serial evaluation of urinary proteins in dogs with glomerular disease that correlates findings with clinical data, and results will provide a foundation for future evaluation of proteinuria-associated CKD in the dog. They will also provide a baseline to conduct studies that correlate proteinuria with protein and mRNA expression in renal tissue from XLHN dogs and eventually from clinical patients,

as histologic examination of renal biopsies becomes more commonplace in the clinical setting.

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APPENDIX A

Stability results for urinary N-acetyl-β-D-glucosaminidase (NAG) and neutrophil gelatinase-associated lipocalin (NGAL) using canine urine.

Table A-1. Stability of urinary NAG (U/L) at room temperature. All samples demonstrated a significant increase in NAG activity over time.

Hours	0	2	4	8	12	24
Dog 1	21.4	22.6	21.3	25.1	27.6	33.8
Dog 2	3	3.3	3.9	4	4.4	5.5
Dog 3	8.8	9.2	10.2	11.2	13.4	15.4
Dog 4	16.4	17.3	18.4	19.9	20.1	25.5

Table A-2. Stability of urinary NAG (U/L) at 4^{o}C. A small but significant increase in NAG activity was observed in one sample.

Weeks	0	2	4	8
Dog 1	21.4	24	22.3	25.8
Dog 2	3	3.4	1.5	2.8
Dog 3	16.4	14.7	14.8	15.7
Dog 4*	2.3	2.4	2.5	2.7

*$P < 0.001$

Table A-3. Stability of urinary NAG (U/L) at -20^{o}C. No significant change in activity was observed over two months.

Weeks	0	2	4	8
Dog 1	21.4	22.9	19.2	17.9
Dog 2	3	3	1.5	3.8
Dog 3	16.4	15.1	14.4	14.4
Dog 4	2.3	3.5	1.5	3.2

Table A-4. Stability of urinary NAG (U/L) at -80°C. No significant change in activity was observed over two months.

Weeks	0	2	4	8
Dog 1	21.4	21.2	19.3	20
Dog 2	3	3.1	2	2.8
Dog 3	16.4	...	14.3	15
Dog 4	2.3	2.8	1.4	2.8

Table A-5. Stability of urinary NAG and NGAL over multiple freeze-thaw cycles (-80°C). A small but significant increase in NAG activity was observed in one sample. No significant change in concentration was observed for NGAL.

Freeze-thaw cycle	0	1	2	3	4	5
NAG (U/L)						
Dog 1	41	36.4	40.1	42.7	44.3	42.7
Dog 2	16.6	16.5	15.9	17.4	18.1	18.4
Dog 3*	3.7	3.6	4.5	4.9	4.9	5.0
NGAL (pg/ml)						
Dog 1	1188	1102	1124	1245	1284	...
Dog 2	12323	12489	11904	11002	12542	...
Dog 3	140607	143661	146390	148685	139904	...

*P = 0.025

APPENDIX B

Effect of storage time on the concentration/activity of each urinary biomarker. Data for the years 2002 and 2005 is limited and includes only a small portion of the disease process in 1-2 dogs. Therefore, data from these years were not considered in the final interpretation.

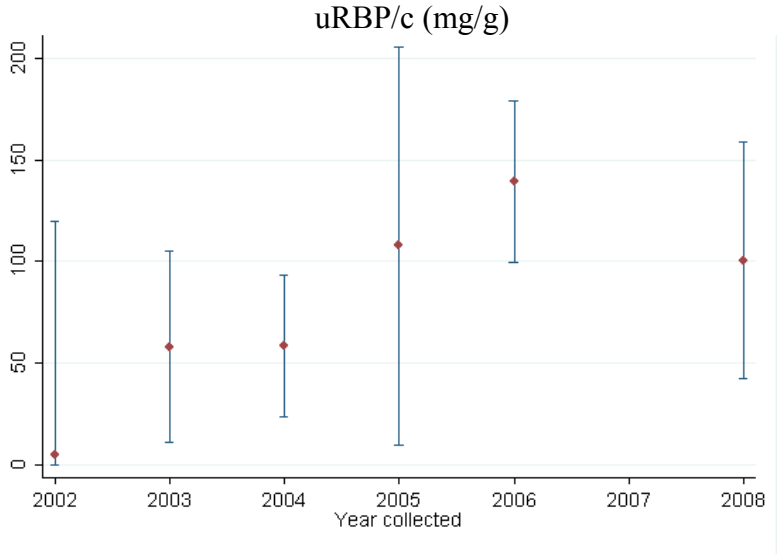

Figure A-1. Plot of mean and standard deviation for uRBP/c for affected XLHN male dogs according to the year of sample collection. A large overlap of the standard deviation is observed for samples collected in 2008 as compared with samples collected in years 2003 and 2004, supporting a lack of storage effect on uRBP determination.

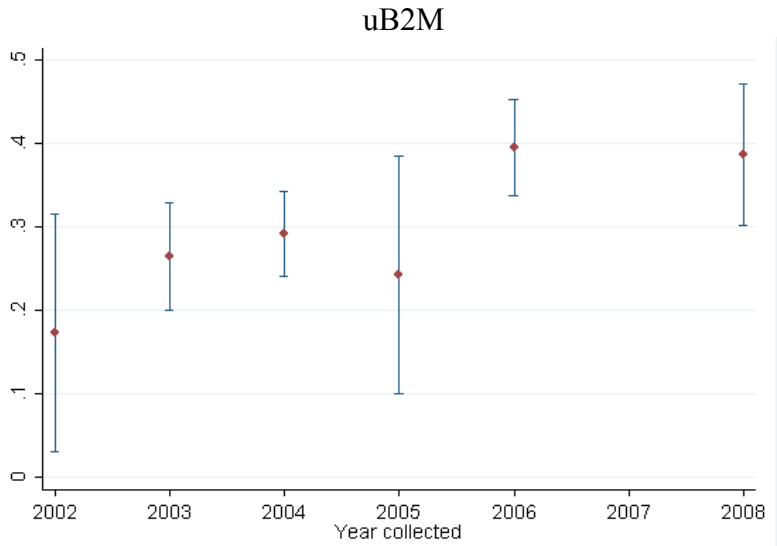

Figure A-2. Plot of mean and standard deviation for uB2M for affected XLHN male dogs according to the year of sample collection. A relatively large degree of overlap in the standard deviation is observed for samples collected in 2008 as compared with samples collected in earlier years, supporting a lack of storage effect on uB2M.

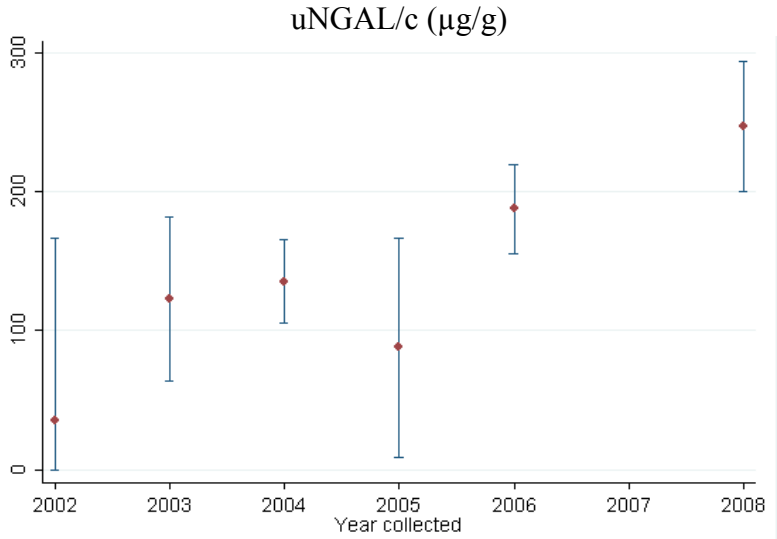

Figure A-3. Plot of mean and standard deviation for uNGAL/c for affected XLHN male dogs according to the year of sample collection. No overlap in the standard deviation is observed for samples collected in 2008 as compared with samples collected in years 2003 and 2004. This supports degradation of uNGAL after 4 years of storage. However, there is no evidence for a substantial decrease from 2006 to years 2003 and 2004, supporting a lack of significant degradation of uNGAL after 2 years of storage.

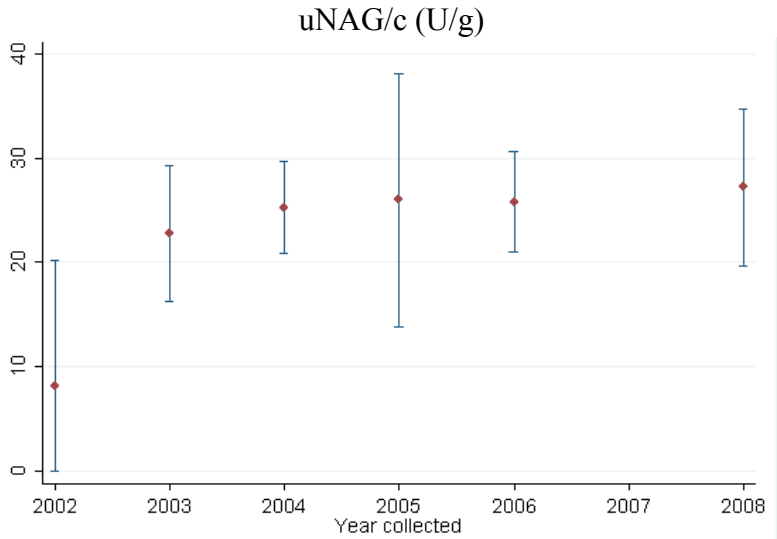

Figure A-4. Plot of mean and standard deviation for uNAG/c for affected XLHN male dogs according to the year of sample collection. Substantial overlap in the standard deviation is observed for samples collected more recently as compared with samples collected in years 2003 and 2004, supporting a lack of storage effect on uNAG determination.

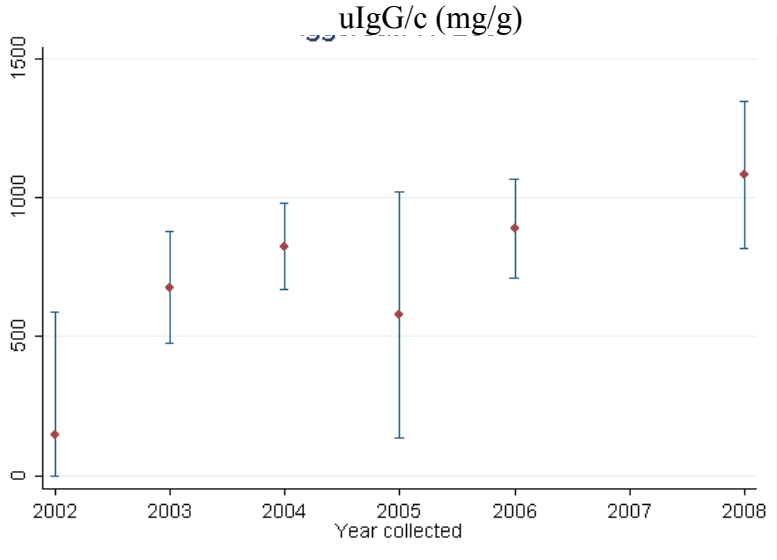

Figure A-5. Plot of mean and standard deviation for uIgG/c for affected XLHN male dogs according to the year of sample collection. A relatively large degree of overlap in the standard deviation is observed for samples collected more recently as compared with samples collected in years 2003 and 2004, supporting a lack of storage effect on uIgG determination.

APPENDIX C

Mean urinary biomarker results for affected XLHN male dogs within defined intervals of serum creatinine, glomerular filtration rate, and urine protein:creatinine ratio, in addition to complete correlation results for histology lesions with clinical and urinary data from all dogs, affected XLHN dogs only, and normal dogs only.

Table A-6. Mean values for urinary biomarkers in male dogs affected with XLHN within defined intervals of conventional clinical measures of renal function.

Creatinine (mg/dL)	sCr	GFR	UPC	uRBP/c (mg/g)	uB2M	uNAG/c (U/g)	uNGAL/c (µg/g)	uIgG/c (mg/g)
<0.6	0.46	4.3	1.3	1.6	0.08	13.3	71.1	76.9
0.6-1.2	0.77	3.5	7.5	10.3	0.26	17.2	138	526
1.2-2.4	1.6	2.2	16.3	67.3	0.49	35.4	240	1359
2.4-6	3.8	1.3	17.5	354	0.53	38.4	249	1620
GFR (ml/min/kg)	**sCr**	**GFR**	**UPC**	**uRBP/c (mg/g)**	**uB2M**	**uNAG/c (U/g)**	**uNGAL/c (µg/g)**	**uIgG/c (mg/g)**
>3.5	0.65	4.2	6.85	8.74	0.27	20.1	163	449
2.5-3.5	0.95	3.0	11.6	25.3	0.33	23.6	184	838
1.5-2.5	2.26	2.0	17.7	143	0.49	41.2	248	1552
<1.5	3.94	1.1	17.74	333	0.52	38.8	229	1398
UPC	**sCr**	**GFR**	**UPC**	**uRBP/c (mg/g)**	**uB2M**	**uNAG/c (U/g)**	**uNGAL/c (µg/g)**	**uIgG/c (mg/g)**
<0.5	0.55	4.6	0.23	0.16	0.01	8.5	18.0	10.2
0.5-2	0.57	4.1	0.97	0.44	0.03	8.2	44.6	50.6
2-10	1.1	3.5	6.2	16.7	0.27	14.8	112.3	397
>10	2.2	2.3	17.1	143.2	0.48	35.4	254	1310

sCr = serum creatinine; GFR = glomerular filtration rate; UPC = urine protein:creatinine ratio; uRBP/c = urine retinol binding protein:creatinine ratio; uB2M = urinary β2-microglobulin; uNAG/c = urine N-acetyl-β-D-glucosaminidase:creatinine ratio; uNGAL/c = urine neutrophil gelatinase-associated lipocalin:creatinine ratio; uIgG/c = urine immunoglobulin G:creatinine ratio.

Histologic correlations: Glomerular lesions

Table A-7. Correlation of glomerular lesions with one other in all dogs (normal and dogs affected with XLHN).

Glomerular lesions	Normal	Seg mes exp	Global mes exp	Obs	Synechia	Fib crescents	BC dilation	Normal & mild
Normal	1.000	…	…	…	…	…	…	…
Seg mes exp	-0.756*	1.000	…	…	…	…	…	…
Glob mes exp	-0.682*	0.239	1.000	…	…	…	…	…
Obs	-0.545*	0.343	0.356	1.000	…	…	…	…
Synechia	-0.864*	0.756*	0.525*	0.716*	1.000	…	…	…
Fib crescents	-0.767*	0.593*	0.435[t]	0.329	0.649*	1.000	…	…
BC dilation	-0.792*	0.442[t]	0.670*	0.127	0.522*	0.555*	1.000	…
Normal & mild	0.957*	-0.534*	-0.775*	-0.552*	-0.782*	-0.729*	-0.827*	1.000
Moderate & severe	-0.753*	0.342	0.878*	0.761*	0.732*	0.471[t]	0.530*	-0.822[t]

[t]P < 0.05; *P < 0.001

Seg mes exp = segmental mesangial expansion; Glob mes exp = global mesangial expansion; Obs = obsolescence; Fib crescents = fibrinous crescents; BC dilation = Bowman's capsule dilation; Normal & mild = normal + segmental mesangial expansion; Moderate & severe = global mesangial expansion + obsolescence.

Table A-8. Correlation of glomerular lesions with one other in dogs affected with XLHN.

Glomerular lesions	Normal	Seg mes exp	Global mes exp	Obs	Synechia	Fib crescents	BC dilation	Normal & mild
Normal	1.000	…	…	…	…	…	…	…
Seg mes exp	-0.610[t]	1.000	…	…	…	…	…	…
Glob mes exp	-0.510	-0.085	1.000	…	…	…	…	…
Obs	-0.415	0.162	0.195	1.000	…	…	…	…
Synechia	-0.757*	0.648[t]	0.287	0.658[t]	1.000	…	…	…
Fib crescents	-0.612[t]	0.405	0.179	0.125	0.424	1.000	…	…
BC dilation	-0.651[t]	0.165	0.538	-0.155	0.210	0.307	1.000	…
Normal & mild	0.916*	-0.242	-0.668[t]	-0.426	-0.599[t]	-0.545	-0.713*	1.000
Moderate & severe	-0.603[t]	0.035	0.821*	0.720*	0.587[t]	0.200	0.290	-0.721*

[t]P < 0.05; *P < 0.001

Seg mes exp = segmental mesangial expansion; Glob mes exp = global mesangial expansion; Obs = obsolescence; Fib crescents = fibrinous crescents; BC dilation = Bowman's capsule dilation; Normal & mild = normal + segmental mesangial expansion; Moderate & severe = global mesangial expansion + obsolescence.

Table A-9. Correlation of glomerular lesions with one other in normal dogs.

Glomerular lesions	Normal	Seg mes exp	Global mes exp	Obs	Synechia	Fib crescents	BC dilation	Normal & mild
Normal	1.000	…	…	…	…	…	…	…
Seg mes exp	-0.569[t]	1.000	…	…	…	…	…	…
Glob mes exp	-0.524[t]	-0.159	1.000	…	…	…	…	…
Obs	NA	NA	NA	…	…	…	…	…
Synechia	NA	NA	NA	NA	…	…	…	…
Fib crescents	-0.233	-0.072	0.340	NA	NA	1.000	…	…
BC dilation	-0.403	-0.184	0.072	NA	NA	-0.084	1.000	…
Normal & mild	0.683*	0.212	-0.764*	NA	NA	-0.340	-0.642*	1.000
Moderate & severe	-0.524[t]	-0.159	1.000*	NA	NA	0.340	0.072	-0.764*

[t]$P < 0.05$; *$P < 0.001$

Seg mes exp = segmental mesangial expansion; Glob mes exp = global mesangial expansion; Obs = obsolescence; Fib crescents = fibrinous crescents; BC dilation = Bowman's capsule dilation; Normal & mild = normal + segmental mesangial expansion; Moderate & severe = global mesangial expansion + obsolescenc; NA = lesion not found.

Histologic correlations: Tubulointerstitial lesions

Table A-10. Correlation of tubulointerstitial lesions with one other in all dogs (normal and dogs affected with XLHN).

TI lesions	Dilation	Lack BB	Necr	Casts	Vac	Atroph	Fibrosis	Min	Chron inflam	Acute inflam
Dilation	1.000	…	…	…	…	…	…	…	…	…
Lack BB	0.746*	1.000	…	…	…	…	…	…	…	…
Necr	0.674*	0.914*	1.000	…	…	…	…	…	…	…
Casts	0.537*	0.729*	0.785*	1.000	…	…	…	…	…	…
Vac	0.101	0.286	0.395	0.234	1.000	…	…	…	…	…
Atroph	0.686*	0.869*	0.841*	0.836*	0.150	1.000	…	…	…	…
Fibrosis	0.775*	0.932*	0.897*	0.734*	0.185	0.915*	1.000	…	…	…
Min	0.510[t]	0.637*	0.499[t]	0.288	0.014	0.450[t]	0.620*	1.000	…	…
Chronic inflam	0.623*	0.902*	0.955*	0.785*	0.352	0.831*	0.871*	0.501[t]	1.000	…
Acute inflam	0.321	0.438[t]	0.401	0.234	0.060	0.373	0.401	0.202	0.431	1.000
Overall Fib	0.792*	0.878*	0.796*	0.743*	0.033	0.909*	0.949*	0.589*	0.788*	0.356

[t]$P < 0.05$; *$P < 0.001$

Dilation = tubular dilation; Lack BB = lack of brush border; Necr = single cell tubular necrosis; Casts = tubular casts; Vac = tubular cell vacuolation; Atroph = tubular cell atrophy; Fibrosis = interstitial fibrosis determined by random fields; Min = tubular basement membrane mineralization; Chronic inflam = chronic interstitial inflammation; Acute inflam = acute interstitial inflammation; Overall Fib = interstitial fibrosis determined from overall estimate of fibrosis using trichrome-stained slides.

Table A-11. Correlation of tubulointerstitial lesions with one other in dogs affected with XLHN.

TI lesions	Dilation	Lack BB	Necr	Casts	Vac	Atroph	Fibrosis	Min	Chron inflam	Acute inflam
Dilation	1.000	…	…	…	…	…	…	…	…	…
Lack BB	0.827*	1.000	…	…	…	…	…	…	…	…
Necr	0.692[t]	0.840*	1.000	…	…	…	…	…	…	…
Casts	0.496	0.612	0.719[t]	1.000	…	…	…	…	…	…
Vac	-0.213	0.030	0.247	0.084	1.000	…	…	…	…	…
Atroph	0.718[t]	0.798*	0.759*	0.772*	-0.123	1.000	…	…	…	…
Fibrosis	0.888*	0.890*	0.828*	0.616	-0.114	0.865*	1.000	…	…	…
Min	0.491	0.567	0.347	0.110	-0.183	0.301	0.535	1.000	…	…
Chronic inflam	0.637[t]	0.810*	0.923*	0.714[t]	0.151	0.727[t]	0.755*	0.355	1.000	…
Acute inflam	0.252	0.341	0.275	0.101	-0.065	0.268	0.297	0.101	0.337	1.000
Overall Fib	0.874*	0.825*	0.680[t]	0.639[t]	-0.293	0.8629*	0.9278*	0.488	0.662	0.239

[t]P < 0.05; *P < 0.001

Dilation = tubular dilation; Lack BB = lack of brush border; Necr = single cell tubular necrosis; Casts = tubular casts; Vac = tubular cell vacuolation; Atroph = tubular cell atrophy; Fibrosis = interstitial fibrosis determined by random fields; Min = tubular basement membrane mineralization; Chronic inflam = chronic interstitial inflammation; Acute inflam = acute interstitial inflammation; Overall Fib = interstitial fibrosis determined from overall estimate of fibrosis using trichrome-stained slides.

Table A-12. Correlation of tubulointerstitial lesions with one other in normal dogs.

TI lesions	Dilation	Lack BB	Necr	Casts	Vac	Atroph	Fibrosis	Min	Chron inflam	Acute inflam
Dilation	1.000	…	…	…	…	…	…	…	…	…
Lack BB	0.147	1.000	…	…	…	…	…	…	…	…
Necr	0.334	0.345	1.000	…	…	…	…	…	…	…
Casts	-0.024	0.066	-0.035	1.000	…	…	…	…	…	…
Vac	0.283	0.228	0.068	-0.102	1.000	…	…	…	…	…
Atroph	-0.025	0.481	0.336	0.450	0.160	1.000	…	…	…	…
Fibrosis	-0.098	0.476	0.337	0.238	0.152	0.913*	1.000	…	…	…
Min	NA	NA	NA	NA	NA	NA	NA	…	…	…
Chronic inflam	-0.032	0.510	0.247	0.234	0.102	0.805*	0.883*	NA	1.000	…
Acute inflam	0.129	0.181	0.262	-0.098	-0.039	-0.065	0.004	NA	0.102	1.000
Overall Fib	0.223	0.110	0.321	0.190	-0.178	0.003	0.195	NA	0.337	0.424

*P < 0.001

Dilation = tubular dilation; Lack BB = lack of brush border; Necr = single cell tubular necrosis; Casts = tubular casts; Vac = tubular cell vacuolation; Atroph = tubular cell atrophy; Fibrosis = interstitial fibrosis determined by random fields; Min = tubular basement membrane mineralization; Chronic inflam = chronic interstitial inflammation; Acute inflam = acute interstitial inflammation; Overall Fib = interstitial fibrosis determined from overall estimate of fibrosis using trichrome-stained slides.

Histologic correlations: Glomerular and tubulointerstitial lesions

Table A-13. Correlation of glomerular lesions with tubulointerstitial lesions in all dogs (normal and dogs affected with XLHN).

	Normal	Seg mes exp	Global mes exp	Obs	Synechia	Fib crescents	BC dilation	Normal & mild	Mod & severe
Dilation	-0.658*	0.278	0.754*	0.404	0.501[t]	0.385	0.605*	-0.721*	0.732*
Lack BB	-0.898*	0.587*	0.751*	0.575*	0.799*	0.614*	0.755*	-0.897*	0.818*
Necr	-0.955*	0.638*	0.704*	0.614*	0.843*	0.731*	0.786*	-0.948*	0.806*
Casts	-0.710*	0.460[t]	0.517[t]	0.854*	0.798*	0.416	0.442[t]	-0.712*	0.800*
Vac	-0.442[t]	0.522[t]	0.023	0.221	0.412	0.530*	0.256	-0.344	0.130
Atroph	-0.784*	0.419	0.712*	0.697*	0.789*	0.396	0.643*	-0.822*	0.854*
Fibrosis	-0.860*	0.443[t]	0.831*	0.564*	0.754*	0.539*	0.793*	-0.908*	0.868*
Min	-0.491[t]	0.335	0.627*	0.083	0.435[t]	0.328	0.616*	-0.484	0.478[t]
Chronic inflam	-0.928*	0.676*	0.714*	0.618*	0.832*	0.692*	0.740*	-0.897*	0.814*
Acute inflam	-0.377	0.185	0.436[t]	0.160	0.314	0.259	0.390	-0.403	0.385
Overall Fib	-0.775*	0.399	0.814*	0.570*	0.720*	0.419	0.679*	-0.819*	0.859*

[t]$P < 0.05$; *$P < 0.001$

Dilation = tubular dilation; Lack BB = lack of brush border; Necr = single cell tubular necrosis; Casts = tubular casts; Vac = tubular cell vacuolation; Atroph = tubular cell atrophy; Fibrosis = interstitial fibrosis determined by random fields; Min = tubular basement membrane mineralization; Chronic inflam = chronic interstitial inflammation; Acute inflam = acute interstitial inflammation; Overall Fib = interstitial fibrosis determined from overall estimate of fibrosis using trichrome-stained slides; Seg mes exp = segmental mesangial expansion; Glob mes exp = global mesangial expansion; Obs = obsolescence; Fib crescents = fibrinous crescents; BC dilation = Bowman's capsule dilation; Normal & mild = normal + segmental mesangial expansion; Mod & severe = global mesangial expansion + obsolescence.

Table A-14. Correlation of glomerular lesions with tubulointerstitial lesions in dogs affected with XLHN.

	Normal	Seg mes exp	Global mes exp	Obs	Synechia	Fib crescents	BC dilation	Normal & mild	Mod & severe
Dilation	-0.679[t]	0.055	0.789*	0.331	0.394	0.209	0.584	-0.783*	0.758*
Lack BB	-0.804*	0.311	0.621	0.446	0.636[t]	0.316	0.590	-0.809*	0.708[t]
Necr	-0.913*	0.382	0.516	0.512	0.706[t]	0.509	0.643[t]	-0.904*	0.673[t]
Casts	-0.589	0.247	0.330	0.831*	0.715[t]	0.132	0.182	-0.583	0.732*
Vac	-0.323	0.482	-0.323	0.105	0.311	0.526	0.062	-0.152	-0.167
Atroph	-0.656[t]	0.125	0.578	0.620	0.663[t]	0.038	0.443	-0.721[t]	0.782*
Fibrosis	-0.758*	0.107	0.757*	0.430	0.570	0.219	0.665[t]	-0.853*	0.795*
Min	-0.334	0.152	0.555	-0.093	0.257	0.119	0.525	-0.325	0.338
Chronic inflam	-0.861*	0.488	0.543	0.523	0.689[t]	0.435	0.548	-0.791*	0.699[t]
Acute inflam	-0.237	0.021	0.337	0.039	0.163	0.087	0.287	-0.273	0.262
Overall Fib	-0.647[t]	0.096	0.7415*	0.445	0.551	0.090	0.508	-0.725[t]	0.793*

[t]$P < 0.05$; *$P < 0.001$

Dilation = tubular dilation; Lack BB = lack of brush border; Necr = single cell tubular necrosis; Casts = tubular casts; Vac = tubular cell vacuolation; Atroph = tubular cell atrophy; Fibrosis = interstitial fibrosis determined by random fields; Min = tubular basement membrane mineralization; Chronic inflam = chronic interstitial inflammation; Acute inflam = acute interstitial inflammation; Overall Fib = interstitial fibrosis determined from overall estimate of fibrosis using trichrome-stained slides; Seg mes exp = segmental mesangial expansion; Glob mes exp = global mesangial expansion; Obs = obsolescence; Fib crescents = fibrinous crescents; BC dilation = Bowman's capsule dilation; Normal & mild = normal + segmental mesangial expansion; Mod & severe = global mesangial expansion + obsolescence.

Table A-15. Correlation of glomerular lesions with tubulointerstitial lesions in normal dogs.

	Normal	Seg mes exp	Global mes exp	Obs	Synechia	Fib crescents	BC dilation	Normal & mild	Mod & severe
Dilation	-0.259	0.032	0.334	NA	NA	0.117	-0.016	-0.280	0.334
Lack BB	-0.356	0.201	0.325	NA	NA	0.217	0.126	-0.245	0.325
Necr	-0.342	0.152	0.502	NA	NA	0.249	-0.078	-0.271	0.502
Casts	0.202	-0.197	0.001	NA	NA	-0.068	-0.013	0.065	0.001
Vac	-0.199	0.241	0.097	NA	NA	-0.027	-0.009	-0.023	0.097
Atroph	-0.055	-0.225	0.409	NA	NA	-0.046	0.244	-0.266	0.409
Fibrosis	-0.124	-0.143	0.424	NA	NA	0.003	0.242	-0.275	0.424
Min	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chronic inflam	-0.064	-0.162	0.358	NA	NA	0.071	0.185	-0.220	0.358
Acute inflam	-0.186	-0.127	0.321	NA	NA	0.697*	-0.024	-0.334	0.321
Overall Fib	-0.159	0.055	0.197	NA	NA	0.645[t]	-0.126	-0.148	0.197

[t]P < 0.05; *P < 0.001

Dilation = tubular dilation; Lack BB = lack of brush border; Necr = single cell tubular necrosis; Casts = tubular casts; Vac = tubular cell vacuolation; Atroph = tubular cell atrophy; Fibrosis = interstitial fibrosis determined by random fields; Min = tubular basement membrane mineralization; Chronic inflam = chronic interstitial inflammation; Acute inflam = acute interstitial inflammation; Overall Fib = interstitial fibrosis determined from overall estimate of fibrosis using trichrome-stained slides; Seg mes exp = segmental mesangial expansion; Glob mes exp = global mesangial expansion; Obs = obsolescence; Fib crescents = fibrinous crescents; BC dilation = Bowman's capsule dilation; Normal & mild = normal + segmental mesangial expansion; Mod & severe = global mesangial expansion + obsolescence.

Histologic correlations: Clinical data and urinary biomarkers

Table A-16. Correlation of glomerular and tubulointerstitial lesions with clinical data and urinary biomarkers in all dogs (normal and dogs affected with XLHN).

	sCr	GFR	UPC	USG	RBP	B2M	NGAL	NAG	IgG
Normal	-0.624*	0.667*	-0.914*	0.394	-0.519	-0.922*	-0.797*	-0.725*	-0.802*
Seg mes exp	0.301	-0.345	0.720*	-0.399	0.216	0.657*	0.529[t]	0.593*	0.584*
Global mes exp	0.661*	-0.674*	0.694*	-0.138	0.680*	0.690*	0.579[t]	0.557[t]	0.616*
Obs	0.613*	-0.608*	0.429	-0.107	0.337	0.523[t]	0.198	0.385	0.237
Synechia	0.619*	-0.608*	0.750*	-0.329	0.404	0.795*	0.484	0.681*	0.577*
Fib crescents	0.329	-0.328	0.703*	-0.166	0.349	0.752*	0.716*	0.570*	0.568*
BC dilation	0.525*	-0.518[t]	0.762*	-0.439	0.440	0.783*	0.892*	0.651*	0.828*
Normal & mild	-0.673*	0.702*	-0.860*	0.330	-0.560[t]	-0.912*	-0.794*	-0.686*	-0.774*
Mod & severe	0.774*	-0.781*	0.704*	-0.151	0.653*	0.7458*	0.485	0.578*	0.547[t]
Dilation	0.642*	-0.631*	0.640*	-0.055	0.671*	0.612*	0.475	0.499[t]	0.607*
Lack BB	0.766*	-0.750*	0.848*	-0.365	0.607[t]	0.846*	0.675*	0.655*	0.781*
Necr	0.723*	-0.692*	0.869*	-0.393	0.530	0.918*	0.735*	0.742*	0.748*
Casts	0.765*	-0.730*	0.607*	-0.297	0.467	0.631*	0.341	0.560[t]	0.456
Vac	0.134	-0.101	0.302	-0.124	-0.031	0.377	0.224	0.331	0.155
Atroph	0.744*	-0.748*	0.717*	-0.320	0.513	0.773*	0.552[t]	0.642*	0.649*
Fibrosis	0.834*	-0.789*	0.830*	-0.336	0.713*	0.850*	0.672*	0.698*	0.764*
Min	0.541*	-0.406	0.531[t]	-0.285	0.498	0.449	0.463	0.430	0.532[t]
Chronic inflam	0.696*	-0.736*	0.889*	-0.463[t]	0.559	0.894*	0.686*	0.744*	0.773*
Acute inflam	0.278	-0.355	0.301	-0.065	0.098	0.342	0.232	0.247	0.428
Overall Fib	0.819*	-0.793*	0.789*	-0.273	0.752*	0.757*	0.612*	0.648*	0.741*

[t]$P < 0.05$; *$P < 0.001$

Seg mes exp = segmental mesangial expansion; Glob mes exp = global mesangial expansion; Obs = obsolescence; Fib crescents = fibrinous crescents; BC dilation = Bowman's capsule dilation; Normal & mild = normal + segmental mesangial expansion; Mod & severe = global mesangial expansion + obsolescence; Dilation = tubular dilation; Lack BB = lack of brush border; Necr = single cell tubular necrosis; Casts = tubular casts; Vac = tubular cell vacuolation; Atroph = tubular cell atrophy; Fibrosis = interstitial fibrosis determined by random fields; Min = tubular basement membrane mineralization; Chronic inflam = chronic interstitial inflammation; Acute inflam = acute interstitial inflammation; Overall Fib = interstitial fibrosis determined from overall estimate of fibrosis using trichrome-stained slides; sCr = serum creatinine; GFR = glomerular filtration rate; UPC = urine protein:creatinine ratio; USG = urine specific gravity; RBP = urine retinol binding protein:creatinine ratio; B2M = urinary β2-microglobulin; NGAL = urine neutrophil gelatinase-associated lipocalin:creatinine ratio; NAG = urine N-acetyl-β-D-glucosaminidase:creatinine ratio; IgG = urine immunoglobulin G:creatinine ratio.

Table A-17. Correlation of glomerular and tubulointerstitial lesions with clinical data and urinary biomarkers in dogs affected with XLHN.

	sCr	GFR	UPC	USG	RBP	B2M	NGAL	NAG	IgG
Normal	-0.547	0.697[t]	-0.821*	0.136	-0.440	-0.856*	-0.656	-0.609	-0.660[t]
Seg mes exp	0.093	-0.195	0.572	-0.287	0.086	0.447	0.329	0.469	0.382
Global mes exp	0.604[t]	-0.692[t]	0.558	0.154	0.649[t]	0.589	0.375	0.455	0.471
Obs	0.546	-0.588	0.227	0.105	0.268	0.382	-0.085	0.238	0.005
Synechia	0.518	-0.571	0.526	-0.085	0.292	0.627[t]	0.172	0.525	0.303
Fib crescents	0.120	-0.165	0.491	0.181	0.239	0.591	0.554	0.385	0.337
BC dilation	0.395	-0.431	0.600	-0.315	0.346	0.666[t]	0.858*	0.534	0.743*
Normal & mild	-0.622[t]	0.748*	-0.717*	0.020	-0.494	-0.847*	-0.660	-0.537	-0.618[t]
Mod & severe	0.745*	-0.830*	0.527	0.169	0.610	0.630[t]	0.195	0.449	0.331
Dilation	0.705[t]	-0.753*	0.669[t]	0.059	0.713[t]	0.656[t]	0.431	0.489	0.605
Lack BB	0.723[t]	-0.782*	0.699[t]	-0.171	0.522	0.708[t]	0.447	0.430	0.625
Necr	0.670[t]	-0.753*	0.710	-0.275	0.416	0.838*	0.521	0.607	0.547
Casts	0.706[t]	-0.712[t]	0.387	-0.089	0.369	0.456	0.044	0.409	0.216
Vac	-0.025	0.074	0.089	-0.171	-0.129	0.225	0.092	0.110	-0.075
Atroph	0.675[t]	-0.746*	0.523	-0.139	0.414	0.655[t]	0.287	0.490	0.460
Fibrosis	0.817*	-0.830*	0.691[t]	-0.120	0.659[t]	0.754*	0.451	0.559	0.609
Min	0.450	-0.326	0.402	-0.221	0.440	0.296	0.333	0.313	0.423
Chronic inflam	0.625	-0.798*	0.761*	-0.317	0.456	0.790*	0.420	0.603	0.589
Acute inflam	0.163	-0.282	0.129	-0.013	-0.005	0.227	0.099	0.118	0.325
Overall Fib	0.775*	-0.810*	0.667[t]	-0.067	0.707[t]	0.630	0.390	0.516	0.613

[t]$P < 0.05$; *$P < 0.001$

Seg mes exp = segmental mesangial expansion; Glob mes exp = global mesangial expansion; Obs = obsolescence; Fib crescents = fibrinous crescents; BC dilation = Bowman's capsule dilation; Normal & mild = normal + segmental mesangial expansion; Mod & severe = global mesangial expansion + obsolescence; Dilation = tubular dilation; Lack BB = lack of brush border; Necr = single cell tubular necrosis; Casts = tubular casts; Vac = tubular cell vacuolation; Atroph = tubular cell atrophy; Fibrosis = interstitial fibrosis determined by random fields; Min = tubular basement membrane mineralization; Chronic inflam = chronic interstitial inflammation; Acute inflam = acute interstitial inflammation; Overall Fib = interstitial fibrosis determined from overall estimate of fibrosis using trichrome-stained slides; sCr = serum creatinine; GFR = glomerular filtration rate; UPC = urine protein:creatinine ratio; USG = urine specific gravity; RBP = urine retinol binding protein:creatinine ratio; B2M = urinary β2-microglobulin; NGAL = urine neutrophil gelatinase-associated lipocalin:creatinine ratio; NAG = urine N-acetyl-β-D-glucosaminidase:creatinine ratio; IgG = urine immunoglobulin G:creatinine ratio.

Table A-18. Correlation of glomerular and tubulointerstitial lesions with clinical data and urinary biomarkers in normal dogs.

	sCr	GFR	UPC	USG	RBP	B2M	NGAL	NAG	IgG
Normal	-0.031	0.113	0.301	0.066	0.420	0.167	0.484	0.178	0.186
Seg mes exp	0.108	-0.135	-0.229	-0.025	-0.230	-0.050	-0.352	-0.125	-0.405
Global mes exp	-0.042	0.146	-0.182	0.057	-0.355	-0.144	-0.206	-0.219	-0.039
Obs	NA	NA	NA	NA	NA	NA	NA	NA	NA
Synechia	NA	NA	NA	NA	NA	NA	NA	NA	NA
Fib crescents	0.276	NA	-0.080	0.156	NA	-0.054	-0.130	-0.104	-0.196
BC dilation	-0.064	-0.233	-0.037	-0.206	-0.071	-0.078	-0.186	0.120	0.245
Normal & mild	0.060	0.003	0.155	0.056	0.329	0.154	0.285	0.106	-0.123
Mod & severe	-0.042	0.146	-0.182	0.057	-0.355	-0.144	-0.206	-0.219	-0.039
Dilation	-0.384	0.315	-0.134	0.226	0.357	0.347	0.014	0.082	0.037
Lack BB	0.155	-0.240	0.030	0.004	0.409	-0.092	-0.178	0.074	-0.225
Necr	0.091	0.272	-0.323	0.425	-0.582	-0.065	-0.088	-0.178	-0.213
Casts	0.224	-0.317	-0.185	-0.331	-0.234	-0.108	-0.170	-0.041	-0.269
Vac	-0.172	0.010	-0.038	0.231	0.296	0.465	-0.138	0.234	-0.143
Atroph	0.090	-0.216	-0.137	-0.108	-0.234	-0.082	-0.192	0.027	-0.388
Fibrosis	0.069	-0.179	-0.083	-0.113	-0.102	-0.061	-0.166	0.009	-0.188
Min	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chronic inflam	0.091	-0.262	0.070	-0.208	0.024	-0.080	-0.069	-0.049	0.027
Acute inflam	0.228	-0.066	-0.153	0.358	NA	0.068	-0.177	-0.149	-0.196
Overall Fib	0.295	-0.148	-0.202	0.018	-0.371	-0.091	-0.203	-0.144	-0.266

Seg mes exp = segmental mesangial expansion; Glob mes exp = global mesangial expansion; Obs = obsolescence; Fib crescents = fibrinous crescents; BC dilation = Bowman's capsule dilation; Normal & mild = normal + segmental mesangial expansion; Mod & severe = global mesangial expansion + obsolescence; Dilation = tubular dilation; Lack BB = lack of brush border; Necr = single cell tubular necrosis; Casts = tubular casts; Vac = tubular cell vacuolation; Atroph = tubular cell atrophy; Fibrosis = interstitial fibrosis determined by random fields; Min = tubular basement membrane mineralization; Chronic inflam = chronic interstitial inflammation; Acute inflam = acute interstitial inflammation; Overall Fib = interstitial fibrosis determined from overall estimate of fibrosis using trichrome-stained slides; sCr = serum creatinine; GFR = glomerular filtration rate; UPC = urine protein:creatinine ratio; USG = urine specific gravity; RBP = urine retinol binding protein:creatinine ratio; B2M = urinary β2-microglobulin; NGAL = urine neutrophil gelatinase-associated lipocalin:creatinine ratio; NAG = urine N-acetyl-β-D-glucosaminidase:creatinine ratio; IgG = urine immunoglobulin G:creatinine ratio.

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