Urinary Biomarkers of Renal Disease in Dogs with X-Linked Hereditary Nephropathy

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Background: Sensitive and specific biomarkers for early tubulointerstitial injury are lacking.

Hypothesis: The excretion of certain urinary proteins will correlate with the state of renal injury in dogs with chronic kidney disease.

Animals: Twenty-five male colony dogs affected with X-linked hereditary nephropathy (XLHN) and 19 unaffected male littermates were evaluated.

Methods: Retrospective analysis of urine samples collected every 2–4 weeks was performed. Urine proteins evaluated were retinol binding protein (uRBP/c), β2-microglobulin (uB2M), N-acetyl-β-D-glucosaminidase (uNAG/c), neutrophil gelatinase-associated lipocalin (uNGAL/c), and immunoglobulin G (uIgG/c). Results were correlated with serum creatinine concentration (sCr), glomerular filtration rate (GFR), urine protein : creatinine ratio, and histopathologic analysis of serial renal biopsies. Analytical validation was performed for all assays; uNAG stability was evaluated.

Results: All urinary biomarkers distinguished affected dogs from unaffected dogs early in their disease process, increasing during early and midstages of disease. uRBP/c correlated most strongly with conventional measures of disease severity, including increasing sCr (r = 0.89), decreasing GFR (r = −0.77), and interstitial fibrosis (r = 0.80), P < .001. However, multivariate analysis revealed age, sCr, uIgG/c, and uB2M, but not uRBP/c, as significant independent predictors of GFR (P < .05).

Conclusions and Clinical Importance: All urinary biomarkers were elevated before sCr increased, but typically after proteinuria developed in dogs with progressive glomerular disease because of XLHN. uRBP/c measurement might be promising as a noninvasive tool for diagnosis and monitoring of tubular injury and dysfunction in dogs.

Key words: N-acetyl-β-D-glucosaminidase; Neutrophil gelatinase-associated lipocalin; Retinol binding protein; β2-microglobulin.

Chronic progressive kidney disease (CKD) leading to renal failure is a common cause of morbidity and mortality in dogs, and it is often a result of primary glomerular diseases.2,3 Regardless of the initiating cause of CKD, 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 (TI) damage.6 However, most currently available, noninvasive clinical methods for detecting TI damage are relatively insensitive. In addition, specificity of these tests, particularly urine specific gravity (USG), can be quite poor. There is currently no clinically available, sensitive, and specific noninvasive marker to detect ongoing tubular damage, decreased tubular function, or both that will ultimately lead to end-stage renal disease (ESRD). Therefore, although TI lesions are frequent in dogs,4,5 they often are not recognized clinically until at an advanced stage, when lesions are both severe and irreversible, in which case options for successful treatment are limited. Detection of TI damage and altered function at an earlier stage would permit interventions with renoprotective treatments that may slow renal disease progression, and early detection is essential in evaluating new potential renoprotective treatments.

Evaluation of certain urinary proteins (ie, qualitative assessment of proteinuria) has shown promise in determining the localization and severity of renal damage in people with various forms of CKD and in detecting tubular dysfunction and injury earlier than conventional methods. For example, low molecular weight (LMW) proteins are increased in the urine because of decreased reabsorption by the renal tubules. Therefore,
measurement of these proteins might be useful in the assessment of tubular function and T1 damage. The LMW proteins urinary β2-microglobulin (B2M), α1-microglobulin (α1M), and retinol binding protein (RBP) have 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 in human patients with CKD.6–10 Other proteins that could be useful to evaluate tubular damage include those released from injured tubular cells, such as N-acetyl-β-D-glucosaminidase (NAG), a lysosomal enzyme that has been shown to be more useful than total urinary protein loss in predicting renal disease progression and response to treatment in human patients with several glomerular diseases.11,12

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).13–20 However, the temporal behavior of these urinary biomarkers in naturally occurring progressive renal disease is unknown, because only one limited serial study of some of these biomarkers exists.16 No comprehensive serial evaluation of urinary biomarkers has been performed in dogs with CKD comparing results to those of 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 in dogs is largely unknown. Therefore, the primary objective of this study was to serially evaluate urinary biomarkers in dogs with a single CKD and to compare their behavior with standard measures of renal function and damage. The secondary objective was to validate the assays for canine urine and to investigate the stability of urinary NAG (uNAG) activity in dogs.

Materials and Methods

Dogs

Analyses were performed on stored urine samples collected between 2002 and 2008 from 25 male dogs with X-linked hereditary nephropathy (XLHN) (22 intact; 3 neutered at 14 weeks of age) and 19 unaffected male littermates (16 intact; 3 neutered at 14 weeks of age). The dogs were members of a single family maintained in a colony at Texas A&M University. In this kindred, XLHN is caused by a mutation in the gene encoding the α5 chain of type IV collagen, which is a crucial component of normal glomerular basement membranes (GBM).21 The salient clinical and pathological features of the nephropathy that occurs in male dogs with XLHN include development of marked proteinuria and progressive CKD that results in ESRD at about 1 year of age.22 All puppies produced in the colony were raised by means of a standardized protocol. 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, midstream urine were collected in the morning on a weekly to biweekly basis from the dogs starting at 8 weeks of age until affected dogs reached ESRD or 12 weeks of age (serum creatinine [sCr] >5 mg/dL). sCr concentration was measured by an enzymatic assay.2 USG was measured with a refractometer. A routine dipstick analysis and, for approximately half of the dogs, semiquantitative microalbuminuria dipstick were performed.2b–e Urine was centrifuged (500 × g for 5 minutes) and the supernatant was removed for urine protein and creatinine determination. The remaining supernatant was frozen within 4–6 hours of collection and stored at ~80°C for 0.5–8 years before biomarker determination. Before analysis, samples were thawed and divided into multiple aliquots so that additional freeze-thaw cycles could be avoided. Therefore, samples typically underwent 2 freeze-thaw cycles.

Biomarker Assays

Proteins were measured in the urine from 20 to 25 dogs affected with XLHN and 10–19 unaffected age-matched littermates. In affected dogs, these proteins were measured every 2 weeks typically starting 2 weeks preceding development of proteinuria (urine protein : creatinine ratio, UPC > 0.5) in each dog (generally 8–12 weeks of age) until the onset of azotemia (sCr ≥ 1.2 mg/dL), after which they were measured monthly (average 10 time points per dog). The cutoff for mild azotemia in these dogs was based on the sCr value that could reliably indicate a decrease in glomerular filtration rate (GFR) in the affected dogs as compared with their unaffected littermates. In the unaffected dogs, the proteins were measured at time points corresponding to their affected littermates for uNAG, but for the remaining proteins, they were measured at 4 time points, typically 2–3 months apart, because of the consistently low levels of these proteins detected in their urine. Samples were run in duplicate for each assay.

Urinary neutrophil gelatinase-associated lipocalin (uNGAL), uRBP, and uIgG were determined by sandwich ELISAs.2d,e All assays were used in accordance with the manufacturer’s instructions. Briefly, for RBP and NGAL, microtiter plates had been precoated with affinity-purified antibodies, whereas for IgG, plates were first coated with capture antibody. Samples were diluted as necessary (range 1/5 to 1/4,000), and 100 µL of either sample or provided standards (RBP, 7.8–250 ng/mL; NGAL, 0–400 pg/mL; IgG, 7.8–500 ng/mL) was placed into duplicate wells and incubated for 60 minutes. After several incubation and wash steps, 3,3′,5,5′-tetramethylbenzidine was added, and the reaction was stopped with sulfuric acid. Absorbance was measured at 450 nm (RBP and IgG) or 450 nm using 620 nm as a reference wavelength (NGAL) with a microplate reader.8 The concentration of analyte was interpolated from provided standards by a 4-parameter logistic curve. Concentrations of analyte within each sample were normalized to urine creatinine concentration and expressed as ratios.

Urinary NAG was evaluated by an enzymatic colorimetric assay on an automated chemistry analyzer.1 In this assay, NAG hydrolyzes 2-methoxy-4-(2′-nitrovinyl)-phenol to 2-acetamido-2-deoxy-β-D-glucopyranoside (MNP-GlcNAc) to 2-methoxy-4-(2′-nitrovinyl)-phenol, which is detected by measuring the absorbance at 505 nm. The assay was run in accordance with the manufacturer’s instructions, except that all sample volumes were doubled to 20 µL instead of 10 µL to increase instrument sensitivity. Results were divided by 2 to obtain final activity. Provided controls and internal quality control samples were assayed during each sample run.
To the authors’ knowledge, a quantitative assay for B2M that cross-reacts with the canine protein is not commercially available. Therefore, uB2M was evaluated by Western blot as described previously with a polyclonal rabbit antihuman B2M antibody. Urine samples were normalized to 20 mg/dL creatinine. Purified human B2M (0.05 µg/ lane) was loaded onto each gel to serve as a positive control as well as to allow for semiquantitative assessment of uB2M in each urine sample. Films were scanned and the integrated density for each band was quantified. The integrated density for each urine sample was divided by that from the standard control lane to obtain a semiquantitative, unitless value for uB2M. Because the amount of B2M in the urine samples was often less than that used for the control lane, the values for uB2M appeared to be quantitatively low.

Analytical Validation of IgG, NAG, RBP, and NGAL Assays

Intra- and interassay 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 because of the high cost of and low replicate variability by the NGAL assay. Assay sensitivity was determined for uNAG. For intra- and interassay variability, 3–5 samples with low, middle, and high concentrations of analyte were assayed with 10 repetitions within 1 assay run or in 6–10 consecutive assay runs, respectively. For linearity, 3–5 samples representing low, middle, and high concentrations of analyte were serially diluted. For spiking recovery, a known amount of provided protein standard was added to 3 samples with known concentrations for the RBP, IgG, and NAG assays. In addition, 3 canine urine samples of low, middle, and high concentrations were combined in various combinations for RBP, NGAL, and NAG assays. Assay sensitivity for uNAG was calculated by 10 blank determinations of water. The detection limit was calculated by the mean blank value ± 2.6 × standard deviation, and values below this were considered to be zero.

The effect of freeze-thaw cycles was evaluated for uNAG and uNGAL with 3 samples (low, middle, and high concentrations/activities) by repeatedly thawing, evaluating, and refreezing the same sample stored at −80°C on different days over 2–4 weeks, for a total of 5 and 4 freeze-thaw cycles, respectively. In addition, for uNAG, stability was evaluated at room temperature for 2, 4, 8, 12, and 24 hours and at 4, −20, and −80°C for 2 weeks, 1, 2, and 6 months, and 1 year for samples with low, middle, and high activity. Because of the change in instrumentation, uNAG stability was evaluated with a Sirius chemistry analyzer.

Glomerular Filtration Rate

For 22 affected dogs and 19 unaffected littersmates, dynamic renal scintigraphy with clearance of 99mTc-diethylenetriaminepentaacetic acid (DTPA) was performed to estimate GFR at monthly intervals starting at 9 weeks of age as described previously. Briefly, 99mTc-DTPA was injected through a cephalic vein, and dogs were imaged with 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. GFR was determined much less frequently than other clinical data and often did not occur in the same week as urine sample analyses. Therefore, GFR estimates were calculated at each week of age by presuming that any change (increase or decrease) that occurred during the interval between 2 sequential determinations was linear on a week-to-week basis (ie, a week-to-week weighted average of these 2 measurements was calculated).

Histological Evaluation

Serial renal biopsies obtained from 22 affected dogs and 19 unaffected littersmates were obtained. Initially, dogs (n = 12 affected, 11 unaffected) were biopsied at time points defined by the dog’s age (months 4, 6, 8, and 10). Later, dogs (n = 10 affected, 8 unaffected) were biopsied when the affected dogs 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 sCr ≥ 1.2 mg/dL (azotemia), onset of sCr ≥ 2.4 mg/dL, and onset of sCr > 5 mg/dL (endpoint). These milestones were chosen based on their appropriateness for determining mild azotemia, moderate azotemia and end-stage azotemia (before development of clinical uremia) in this population of dogs. Three to four serial biopsies were obtained from most dogs. Unaffected male littermates were biopsied at time points corresponding with the affected dogs. Biopsies were obtained and processed as described previously. Three serial sections were cut (3 µm thickness) from each biopsy. A second set of serial sections was cut approximately 100 µm deeper into the tissue. Therefore, each biopsy had 2 sets of serial sections spaced 100 µm apart to represent different planes of the tissue. Serial sections were routinely stained with H&E, Masson’s trichrome, and Periodic acid-Schiff. Slides were digitally scanned with a ScanScope CS® for evaluation by a board-certified anatomic pathologist (REC).

To score the glomeruli, the digital images of 3 serial sections (stained with PAS, H&E, and trichrome) were aligned, and all intact glomeruli were evaluated. The percentage of glomeruli with the features listed in Table 1 was determined. To score the tubulointerstitium, the PAS and H&E serial sections were digitally aligned and 20 randomly chosen 200× fields were evaluated. Only the renal cortex was scored (scoring method outlined in Table 2). The scores (0–3) were added together so that the total score for each biopsy time point within each TI category ranged from 0 to 60.

Statistical Methods

Intra- and interassay variability was calculated by coefficient of variation. Dilutional parallelism and spiking recovery were assessed by means of observed to expected ratios. Random effects linear regression was also used to determine dilutional parallelism and to evaluate NAG stability and the effect of freeze-thaw cycles. Simple linear regression with clustered robust standard errors was used to determine the effect of storage time on urinary biomarker determinations; urine samples collected during 2 years (2002 and 2005) were eliminated from this analysis because of the limited number of samples collected during those years. Clinical data for affected and unaffected dogs were compared by means of medians and interquartile ranges (IQR). Comparison of median values between affected and unaffected dogs when sCr ≤ 0.7 mg/dL, when UPC < 0.5, and for each histological category was performed by quartile regression with bootstrapped standard error clustered by dog. Correlations between the urinary biomarkers and clinical and histological variables were determined after transformation with square root or natural log as necessary to improve normality. Correlation coefficients and their corresponding significance were calculated by means of linear regression with clustered robust standard errors. Multivariate analysis was performed by means of linear regression. Residuals were determined to be normally distributed by Shapiro–Wilks. Significance was set at P value < .05, unless otherwise noted. All statistical calculations were performed by Stata 11.0.
Results

**Analytical Validation of IgG, NAG, RBP, and NGAL Assays**

Analytical validation results for each urinary biomarker are presented in Table 3. Mean intra- and interassay variability was acceptable for all assays (<10 and 15%, respectively). However, for the NAG assay, variability for the low activity samples (<5 U/L) was high (13.9 and 15.6%). Linearity for all assays was acceptable (Table 3), although this was true for the RBP assay only when the values obtained fell
between approximately 7.8 and 130.0 ng/mL on the standard curve; when above this value, final 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 acceptable when samples were spiked with NAG control material 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 3).

After storage at RT, a minimal but statistically significant decrease in NAG activity was observed after 12–24 hours for 2 samples (Supporting Information, Fig S1). There was no statistical evidence of an effect of storage temperature on NAG activity for 1 year at –80 or –20°C although a statistically significant, but not clinically significant, increase in NAG activity was observed for 2 of the samples stored at 4°C by 6 months (Fig S2). There was also a statistically significant (P = .05) but clinically insignificant increase in NAG activity after 4 freeze-thaw cycles. However, for uNGAL, the concentration was not significantly affected with up to 4 freeze-thaw cycles (Fig S3). Despite storage of samples for up to 8 years before analysis, no statistically significant storage effect was observed for any of the analytes (Fig S4). However, all but uNGAL/c showed an increasing trend as storage time decreased.

### Comparison of Results for Urinary Biomarkers with Clinical Data

In the unaffected dogs, uIgG/c and uRBP/c were low, and uB2M was typically below the detection limit (Table 4). However, uNGAL/c in unaffected puppies <4 months of age was frequently substantially higher than that in unaffected dogs >4 months of age (data not shown). uNAG/c was generally low in the unaffected males; however, mild to moderate increases were seen relatively frequently at a young age and occasionally at

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### Table 3. Analytical validation results for quantitative assays for urinary biomarkers with canine urine.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Intra-assay CV (%) &lt;sup&gt;a&lt;/sup&gt;</th>
<th>Interassay CV (%) &lt;sup&gt;a&lt;/sup&gt;</th>
<th>Linearity (O/E%) &lt;sup&gt;b&lt;/sup&gt;</th>
<th>Spiking recovery (O/E%) &lt;sup&gt;c&lt;/sup&gt;</th>
<th>Detection limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>7.3</td>
<td>4.5</td>
<td>92–122</td>
<td>86–117</td>
<td>ND</td>
</tr>
<tr>
<td>RBP</td>
<td>6.6</td>
<td>5.6</td>
<td>97–111</td>
<td>70–116</td>
<td>ND</td>
</tr>
<tr>
<td>NAG</td>
<td>7.1</td>
<td>4.7</td>
<td>93–119</td>
<td>96–111&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.4 U/L</td>
</tr>
<tr>
<td>NGAL</td>
<td>ND</td>
<td>ND</td>
<td>96–107</td>
<td>90–101</td>
<td>ND</td>
</tr>
</tbody>
</table>

O/E, observed/expected; CV, coefficient of variation; ND, Not determined.

<sup>a</sup>Mean CV for all samples evaluated.

<sup>b</sup>Range.

<sup>c</sup>Observed/expected% when mixing samples with moderate to high NAG activity (>8 U/L).

<sup>d</sup>Observed/expected% when mixing a sample with low NAG activity (<5 U/L) and a sample with moderate to high NAG activity.

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### Table 4. Group descriptive statistics (median, interquartile range) for clinical data from all unaffected dogs and from unaffected and XLHN dogs when sCr/C20 ≥ 0.7 mg/dL.

<table>
<thead>
<tr>
<th>Group</th>
<th>sCr (mg/dL)</th>
<th>GFR (mL/min/kg)</th>
<th>UPC</th>
<th>uRBP/c (mg/g)</th>
<th>uB2M</th>
<th>uNAG/c (U/g)</th>
<th>uNGAL/c (l/g/g)</th>
<th>uIgG/c (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Ages</td>
<td>0.8 (0.6–0.9)</td>
<td>3.17 (2.93–3.51)</td>
<td>0.08 (0.06–0.14)</td>
<td>0.00 (0–0)</td>
<td>0.4 (0–3.8)</td>
<td>1.6 (0.7–5.9)</td>
<td>2.2 (1.6–3.0)</td>
<td>2.2 (1.6–3.0)</td>
</tr>
<tr>
<td>sCr ≤ 0.7 mg/dL</td>
<td>0.6 (0.5–0.7)</td>
<td>3.38 (3.1–3.78)</td>
<td>0.15 (0.13–0.2)</td>
<td>0.00 (0–0)</td>
<td>1.9 (0–8.4)</td>
<td>7.9 (3.4–18.0)</td>
<td>3.0 (2.7–4.5)</td>
<td>3.0 (2.7–4.5)</td>
</tr>
<tr>
<td>sCr &gt; 0.7 mg/dL</td>
<td>0.6 (0.5–0.6)</td>
<td>3.87 (3.46–4.42)</td>
<td>0.38 (0.13–2.5)</td>
<td>0.01 (0–0.29)</td>
<td>8.1 (3.3–18.9)</td>
<td>34.6 (8.0–107.6)</td>
<td>48.0 (11.1–292)</td>
<td>48.0 (11.1–292)</td>
</tr>
</tbody>
</table>

<sup>*</sup>Affected versus unaffected dogs when sCr ≤ 0.7 mg/dL.
older ages as well (data not shown). For uB2M, a faint band was observed in at least 1 urine sample from 4 of the 18 unaffected male dogs evaluated by Western blot (data not shown). In the unaffected dogs, the urinary biomarkers (except B2M), GFR, and UPC all demonstrated a significant negative correlation with age \( (P < .01) \), and therefore many of these were also correlated with each other and with sCr, which demonstrated a significant positive correlation with age \( (r = 0.85) \).

In the affected dogs, each urinary biomarker was either significantly increased or approaching statistical significance in affected dogs when compared with unaffected dogs early in the disease process, except for uB2M (Table 4). However, this was true for the UPC as well. ulG/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 (Fig 1). However, comparison of the IQR for these biomarkers at the last 2 sCr intervals showed substantial overlap (Fig 1). In contrast, uRBP/c showed a progressive increase at all intervals, most pronounced in the mid- to late stages of renal disease progression. Of note, whereas the ranges for sCr in Figure 1 were chosen based on their appropriateness for this population of dogs, they roughly correspond with the IRIS CKD staging system,\(^p\) with 0.6-1.2 mg/dL corresponding with Stage 1, 1.2-2.4 mg/dL corresponding with Stage 2, and 2.4-6 mg/dL corresponding with Stage 3. When plotted against GFR (Fig 2), results are similar, with each biomarker increasing until a time point of moderate renal impairment, although overlap of the IQR was substantial for all biomarkers except uRBP/c. However, at a time point of end-stage renal impairment, median values for all biomarkers stayed at a similar level or decreased slightly, except for uRBP/c, which continued to show a marked increase. As compared with UPC, most of the urinary biomarkers demonstrated a substantial increase only when UPC > 2 (Fig 3), and particularly marked increases were observed in all biomarkers when UPC > 10, although the increase in median uRBP/c was much less pronounced than that for the other biomarkers. Only uNAG/c was significantly higher in affected dogs as compared with unaffected dogs when dogs were both young (<20 weeks) and had UPC < 0.5 \( (P < .001) \). Although not statistically significant, ulG/c was often increased before UPC > 0.5 in affected dogs.

Correlations of each urinary biomarker with sCr, GFR, UPC, and USG reveal that serum creatinine and GFR correlated most strongly with each other, followed closely by uRBP/c (Fig 4). UPC correlated most strongly with ulG/c, but all biomarkers showed a moderate to high correlation with the magnitude of proteinuria. USG showed the least correlation with conventional clinical data and urinary biomarkers. Correlations of the urinary biomarkers with each other showed uRBP/c, uB2M, uNGAL/c, and ulG/c to all
be strongly correlated with one another ($r = 0.75–0.87$; Supplemental Table S1). uNAG/c demonstrated moderate correlation with the other urinary biomarkers ($r = 0.68–0.73$; Table S1).

**Multivariate Analysis**

Age, sCr, uIgG/c, and uB2M all remained significant variables for the prediction of GFR, with age and sCr having the strongest predictive effect on GFR. The interaction of age and uRBP/c was also marginally significant ($P = .041$). uIgG/c and uB2M contributed different effects. For example, a high uIgG/c at a young age was predictive of a lower GFR, whereas a high uB2M at an older age was predictive of a lower GFR.

**Histological Analysis**

Glomerular lesions were evident earlier than TI lesions in the affected dogs, and glomerular and TI lesions were significantly increased in the affected dogs as compared with their unaffected littermates, except for segmental sclerosis with a normal tuft, glomerular atrophy, glomerular obsolescence, fetal glomeruli, tubular dilatation, tubular epithelial cell microvesicles, and acute interstitial inflammation, although tubular dilatation approached statistical significance ($P = .06$). Of these, the only category more commonly observed in unaffected dogs was fetal glomeruli. In affected dogs, moderate to severe glomerular tuft lesions were associated with Bowman’s capsule (BC) lesions (synechia [$r = 0.90$], crescents [$r = 0.70$], and BC dilation [$r = 0.68$]). Most tubular lesions were strongly and positively correlated with each other, particularly tubular degeneration/regeneration, interstitial fibrosis, and interstitial chronic inflammation ($r = 0.95–0.96$). Tubular epithelial cell microvesicles and an intact brush border demonstrated moderate negative correlation with other TI lesions. Acute inflammation showed weak to minimal correlation with other tubular lesions. Except for tubular microvesicles and acute inflammation, tubular lesions (particularly degeneration/regeneration, fibrosis, and chronic inflammation) demonstrated moderate to strong positive correlation with moderate to severe glomerular lesions and synechiae (data not shown).

When compared with conventional tests of renal function in affected dogs, most glomerular and TI lesions demonstrated a moderate to high correlation with decreasing GFR and increasing sCr and UPC (Table 5). USG showed no significant correlation with any type of glomerular lesion, and it demonstrated the weakest correlation with TI lesions as compared with all other clinical parameters. Few significant correlations ($<0.01$) were observed between the histological lesions and conventional renal tests in unaffected dogs, and all of these correlations were weak ($r < 0.3$).

All urinary biomarkers showed a moderate to high positive correlation with most glomerular and TI lesions (Table 5). uRBP/c demonstrated the strongest correlation with all TI lesions and several of the glomerular lesions as compared with the other urinary biomarkers. It was also the only urinary biomarker to correlate more strongly with severe glomerular and TI damage than conventional measures of renal function.

**Discussion**

In this study, dogs with progressive glomerular disease because of XLHN demonstrated marked increases in all urinary biomarkers evaluated, often very early in their disease process, as compared with unaffected littermates. 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. The pattern of urinary appearance of the proteins did not seem to rely on the proposed mechanism of appearance. Both B2M and RBP originate from filtered plasma and appear in the urine secondary to decreased reabsorption because of
Table 5. Correlation of histological lesions with conventional measures of renal function and urinary biomarkers in dogs with XLHN.

<table>
<thead>
<tr>
<th>Number of observations</th>
<th>GFR</th>
<th>sCr</th>
<th>UPC</th>
<th>USG</th>
<th>uRBP/c</th>
<th>uB2M</th>
<th>uNGAL/c</th>
<th>uNAG/c</th>
<th>uIgG/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0.728**</td>
<td>-0.739**</td>
<td>-0.773**</td>
<td>0.093</td>
<td>-0.802**</td>
<td>-0.749**</td>
<td>-0.658**</td>
<td>-0.649**</td>
<td>-0.721**</td>
</tr>
<tr>
<td>Mild tuft abnormalities</td>
<td>-0.089</td>
<td>-0.306</td>
<td>-0.122</td>
<td>-0.166</td>
<td>-0.225</td>
<td>-0.247</td>
<td>-0.177</td>
<td>0.405</td>
<td>0.114</td>
</tr>
<tr>
<td>Moderate/severe tuft</td>
<td>-0.730**</td>
<td>0.740**</td>
<td>0.807**</td>
<td>-0.191</td>
<td>0.823**</td>
<td>0.741**</td>
<td>0.666**</td>
<td>0.618**</td>
<td>0.746**</td>
</tr>
<tr>
<td>Synechia</td>
<td>-0.723**</td>
<td>0.734**</td>
<td>0.765**</td>
<td>-0.148</td>
<td>0.812**</td>
<td>0.730**</td>
<td>0.564**</td>
<td>0.672**</td>
<td>0.686**</td>
</tr>
<tr>
<td>Fibrinous crescents</td>
<td>-0.323</td>
<td>0.301</td>
<td>0.655</td>
<td>-0.233</td>
<td>0.505</td>
<td>0.623</td>
<td>0.598</td>
<td>0.373</td>
<td>0.619**</td>
</tr>
<tr>
<td>BC dilation</td>
<td>-0.448*</td>
<td>0.467**</td>
<td>0.543**</td>
<td>-0.400</td>
<td>0.553</td>
<td>0.591</td>
<td>0.637</td>
<td>0.432**</td>
<td>0.588**</td>
</tr>
<tr>
<td>No BC space lesions</td>
<td>0.707**</td>
<td>-0.720*</td>
<td>-0.790*</td>
<td>0.251</td>
<td>-0.812</td>
<td>-0.754</td>
<td>-0.667</td>
<td>-0.612*</td>
<td>-0.744**</td>
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<tr>
<td>Tubulointerstitial</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intertstitial fibrosis</td>
<td>-0.768**</td>
<td>0.768**</td>
<td>0.747**</td>
<td>-0.328</td>
<td>0.803**</td>
<td>0.640**</td>
<td>0.608**</td>
<td>0.670**</td>
<td>0.694**</td>
</tr>
<tr>
<td>Chronic inflammation</td>
<td>-0.710**</td>
<td>0.759**</td>
<td>0.758**</td>
<td>-0.359</td>
<td>0.803**</td>
<td>0.660**</td>
<td>0.651**</td>
<td>0.614**</td>
<td>0.713**</td>
</tr>
<tr>
<td>Tubular degeneration/</td>
<td>-0.728**</td>
<td>0.721**</td>
<td>0.754**</td>
<td>-0.372</td>
<td>0.796</td>
<td>0.687</td>
<td>0.634</td>
<td>0.601**</td>
<td>0.715**</td>
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<tr>
<td>regeneration</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Tubular single cell necrosis</td>
<td>-0.535**</td>
<td>0.557**</td>
<td>0.716**</td>
<td>-0.351</td>
<td>0.713**</td>
<td>0.645**</td>
<td>0.585**</td>
<td>0.542**</td>
<td>0.700**</td>
</tr>
<tr>
<td>Tubular atrophy</td>
<td>-0.719**</td>
<td>0.711**</td>
<td>0.612**</td>
<td>-0.265</td>
<td>0.704**</td>
<td>0.534**</td>
<td>0.498**</td>
<td>0.573**</td>
<td>0.565**</td>
</tr>
<tr>
<td>Tubular dilation</td>
<td>-0.682**</td>
<td>0.667**</td>
<td>0.596**</td>
<td>-0.225</td>
<td>0.673**</td>
<td>0.558**</td>
<td>0.416</td>
<td>0.603**</td>
<td>0.512**</td>
</tr>
<tr>
<td>Intact brush border</td>
<td>0.577**</td>
<td>-0.693**</td>
<td>-0.504**</td>
<td>0.051</td>
<td>-0.657**</td>
<td>-0.364</td>
<td>-0.393</td>
<td>-0.587**</td>
<td>-0.484**</td>
</tr>
<tr>
<td>Tubular microvesicles</td>
<td>0.468*</td>
<td>-0.482*</td>
<td>-0.319*</td>
<td>-0.058</td>
<td>-0.514**</td>
<td>-0.215</td>
<td>-0.188</td>
<td>-0.513**</td>
<td>-0.339**</td>
</tr>
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</table>

GFR, glomerular filtration rate; sCr, serum creatinine; UPC, urine protein : creatinine ratio; USG, urine specific gravity; BC, Bowman’s capsule.

*p < .01.

**p < .001.

tubular injury, but only B2M had a similar pattern of urinary appearance as NAG and NGAL, which are both proposed to originate from damaged tubular cells. uRBP/c correlated best with sCr and GFR, and it had the strongest correlation with irreversible histological damage as compared with all other clinical measures. In addition, acceptable assay performance in canine urine was demonstrated by commercially available tests for RBP, NGAL, IgG, and NAG, and this is the 1st report measuring uNGAL in dogs.

The relative absence of the urinary biomarkers in unaffected dogs is consistent with previous studies, and the quantitative values obtained in the present study are similar to those reported in healthy dogs for uNAG/c, uRBP/c, and uIgG/c. The observation of increased uNGAL/c in some unaffected puppies <4 months of age is likely because of the contamination of the urine with preputial leukocytes because it was common to see pyuria in young puppies due to difficulty obtaining a clean midstream urine sample. The transient increases in uNAG/c in the unaffected dogs may be a result of either semen or leukocyte contamination or, less likely, transient tubular damage. Leukocyte-origin effects on uNAG/c and uNGAL/c due to contamination was likely present in young affected males as well, but because similar contamination issues would be expected in both the unaffected and affected males, any differences detected were reasonably assumed to be due to the disease process. As the disease progressed, any effect of contamination was likely negligible given the marked increases observed in these proteins. Although urine culture was not routinely performed, urinary tract infection was considered an unlikely confounding factor in this population based our experience.

In the dogs affected with XLHN, the increase in urinary biomarkers was evident well before an increase in sCr but, except for uNAG/c, a statistically significant increase was not observed before an increase in UPC was observed. This finding would be expected in dogs with primary glomerular disease, because glomerular injury can lead to tubular damage. However, the relative contribution of glomerular as opposed to tubular injury to the urinary biomarker concentrations is difficult to ascertain. Generally it is accepted that an increase in urinary LMW proteins (RBP, B2M) or tubular enzymes (ie, NAG) indicates tubular damage, whereas an increase in IMW and HMW proteins indicates glomerular damage. However, increased filtration of IMW and HMW proteins may interfere with reabsorption of LMW proteins by the renal tubules. In addition, increases in uNAG have been associated with increased lysosomal turnover, which can occur secondary to proteinuria. Therefore, although these urinary proteins all appear promising as markers of TI injury, it may be difficult to determine how much of an increase in the tubular proteins is secondary to glomerular proteinuria as opposed to tubular injury and declining tubular function in patients with marked proteinuria.

Of the proteins evaluated in this study, uRBP/c appeared to be quantitatively the least affected by the magnitude of proteinuria based on Figure 3. Furthermore, one study demonstrated normal uRBP in some human patients with marked proteinuria. These
findings suggest that competition for reabsorption may not be a major factor for the presence of RBP in urine as compared with other LMW proteins (eg, B2M). Furthermore, uRBP/c had the strongest correlation with both clinical and histological evidence of progressive TI damage. It was also one of the more promising proteins identified by proteomic techniques in XLHN dogs with early TI lesions. uRBP/c might therefore be useful for detecting early tubular damage 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 also suggests that it may be useful in monitoring the progression of renal disease to help distinguish between prerenal and renal influences contributing to an increased sCr. Of note, the correlation between uRBP/c and UPC ($r = 0.85$) was slightly weaker than that between uRBP/c and sCr ($r = 0.89$). This contrasts with 2 studies where uRBP/c had a stronger correlation with proteinuria than azotemia in dogs with renal disease. In addition, 1 study found RBP to be insensitive to mild decreases in GFR. Clearly, further investigation of uRBP in dogs is warranted to determine its utility in early detection and monitoring of renal disease.

The remaining urinary biomarkers of tubular function, injury or both (uB2M, uNAG, and uNGAL) all behaved in a similar manner despite the differences in their proposed urinary appearance, and they do not appear to be helpful for monitoring mid- to late-stage renal failure in dogs with XLHN. The relatively constant excretion of uNAG and uNGAL during mid to late-stage disease is not surprising if these proteins indicate a constant level of tubular damage in these dogs. However, both these proteins may also be increased secondary to glomerular proteinuria, because uNAG/c can increase due to proteinuria and subsequent increased lysosomal turnover, and NGAL is a LMW protein that circulates in the blood and may therefore be present in the urine due to increased competition for tubular reabsorption secondary to glomerular proteinuria. Therefore, the contribution of tubular damage to the urinary excretion of these proteins is difficult to assess. Certainly, their correlation with tubular damage is comparable to sCr, GFR, and UPC. The relatively stable excretion of uB2M in mid- to late-stage disease was unexpected, and this may indicate a low threshold for reabsorption that is easily overwhelmed by competition from intermediate to high molecular weight proteins. Of note, urine pH must be considered when evaluating B2M, because its stability can markedly decrease when urine pH $< 6$. However, the urine pH of these dogs almost invariably ranged from 6 to 8, and the consistency in the trends observed for B2M despite urine pH suggests that pH had a minimal effect on B2M results in this study.

The strong correlation of UPC with the single glomerular protein evaluated (IgG) was expected given that both measurements can provide an indication of altered glomerular permselectivity. In addition, uIgG/c did not correlate more strongly than UPC with severity of glomerular damage, indicating that measurement of this protein does not provide significant information beyond that obtained from the UPC in XLHN dogs; however, its utility in dogs with other types of glomerular diseases is unknown. One particular point of interest regarding uIgG/c in these dogs is that it was often increased in the affected dogs as compared with the unaffected dogs even before an increase in UPC, although this finding was not statistically significant. This may seem surprising given that uIgG is typically thought to indicate severe glomerular damage. However, a study in another dog model of XLHN demonstrated focal ultrastructural GBM changes by 1 month of age. Therefore, the early increase in uIgG/c in the current study is likely because of a few scattered but marked GBM alterations that result in some increased passage of proteins (including IgG) but that are not frequent enough to increase the magnitude of proteinuria to the extent that would increase UPC.

The ability to relate both conventional and novel tests of renal function and damage with serial renal biopsies is a unique aspect of this study, and most lesions corresponded as expected with conventional tests of renal function. In addition, all urinary biomarkers demonstrated moderate to strong correlations with most of the histological categories, which may indicate their ability to noninvasively reflect these changes. However, because of their strong correlation with UPC, the influence of proteinuria needs further clarification. As expected, glomerular lesions occurred before TI lesions in these dogs, and moderate to severe glomerular lesions were associated with irreversible TI lesions. The findings also confirm a strong relationship among the hallmark lesions of progressive CKD, including tubular degeneration/regeneration, interstitial fibrosis, and interstitial chronic inflammation. Interestingly, BC dilation was extremely common in this dog population, which may be because of abnormal collagen composition of BC secondary to their genetic mutation.

The present study showed acceptable assay performance for all quantitative assays, with similar precision and linearity results by means of the same assay for uRBP and different assays for uIgG and uNAG as compared with previous studies. In addition, detection limits for uNAG activity were similar. However, in the present study, poor recovery was found 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. The present study also revealed that several dilutions should be used when determining uRBP to ensure accurate results.

One of the major limitations of this study was the variable storage time of the urine before analysis. However, no significant differences were observed when comparing values obtained in older samples to those obtained in newer samples, despite an increasing trend in newer samples for all analytes except NAG. Whether this latter finding 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 storage length on the trends observed. Lastly, the concentrations obtained for both unaffected and affected dogs were similar to those reported by other studies where a comparison was possible.

These findings support that the proteins evaluated in this study are all relatively robust with regard to long-term storage at \(-80^\circ\text{C}\), and that interpretations made from the analysis of these samples are likely to be minimally affected by storage time. However, only the stability of uNAG was specifically addressed in this study, and whereas results support relatively long-term stability of uNAG, similar to previous findings in dogs and people, a recent study found a large decrease in NAG activity in dogs after 12 months of storage at both \(-20\) and \(-80^\circ\text{C}\). Therefore, the effects of long-term storage on uNAG and the other biomarkers needs further investigation. In addition, uNAG in low activity samples may be altered with freeze-thaw cycles indicating that the number of freeze-thaw cycles should be minimized when evaluating uNAG. Another limitation of this study is that a single disease process was evaluated. Although this allowed extensive serial evaluation of many variables, caution should be used before extrapolating results to dogs with other causes of CKD.

The method for GFR determination might be considered a limitation of this study, as renal scintigraphy is not considered the gold standard for GFR estimation. However, the GFR estimates produced in this study were internally consistent throughout the study. For example, serial estimates in individual dogs were consistent with one another, and the estimates obtained among all dogs over the entire course of the study were also consistent with one another. In addition, GFR estimates correlated well with other clinical and clinicopathologic data obtained from these dogs. Therefore, the method for estimating GFR used in this study accurately reflected the GFR trends that occurred in these dogs and is suitable for making comparisons among dogs within this study. The use of interpolated GFR estimates for those time points in between actual GFR determinations is considered a minor limitation given the relative consistency of GFR estimates within a single dog over time.

In summary, all the urinary biomarkers evaluated increased early in the disease process in dogs with XLHN; however, only uRBP/c appears to be strongly correlated with renal disease progression. Therefore, measurement of uRBP/c might be clinically useful for both early detection and monitoring of CKD in dogs, and a canine specific assay for uRBP would be helpful for this purpose. However, future studies are needed to determine if similar trends are seen with CKD due to a variety of other causes. Ultimately, as we work to improve the diagnosis and monitoring of CKD in dogs, determination of the most useful urinary biomarkers will help ensure that this noninvasive, global assessment of kidney function can be used in conjunction with histopathology to create a more complete assessment of renal function, damage, or both.

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**Footnotes**

a Vitros 250, Johnson & Johnson Co, Rochester, NY; uses a pyrocatechol violet-molybdate complex to bind urine proteins
b Multistix, Bayer Corp, Elkhart, IN
c ERD-HealthScreen, Heska Corp, Loveland, CO
d Human Retinol Binding Protein ELISA kit, Immunology Consultants Laboratory Inc, Newberg, OR
e Dog NGAL ELISA kit, BioPorto Diagnostics, Denmark
f Dog IgG ELISA Quantitation Set, Bethyl Laboratories Inc, Montgomery, TX
g BioTek Synergy 2, Winooski, VT
h Diazyme Laboratories, Poway, CA
i Roche Hitachi 911, GM1 Inc, Anoka, MN
j Dako, Carpinteria, CA
k Sigma, St. Louis, MO
l ImageJ, US National Institutes of Health, Bethesda, MD
m Sirrus Clinical Chemistry Analyzer, Stanbio Laboratory, Boerne, TX
n Aperio Technologies Inc, Vista, CA
o Intercooled Stata 11.0, Stata Corp LP, College Station, TX

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**References**


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Plot of urinary NAG activity during storage at room temperature over 24 hours. A minimal but statistically significant decrease in NAG activity was observed after 12–24 hours for 2 samples (represented by the orange and green lines).

Fig S2. Plots of urinary NAG activity during storage at 4, −20, and −80°C over 1 year. A statistically significant, but not clinically significant, increase in NAG activity was observed for 2 of the samples stored at 4°C (represented by the green and blue lines). There was no statistical evidence of an effect of storage temperature on NAG activity for 1 year at −20 or −80°C.

Fig S3. Plot of urinary NAG activity and NGAL concentration over 0–5 and 0–4 freeze-thaw cycles, respectively. There was a statistically significant (P = .05) but clinically insignificant increase in NAG activity after 4 freeze-thaw cycles. The NGAL concentration was not significantly affected with up to 4 freeze-thaw cycles.

Fig S4. Descriptive plots (median, range) and linear regression plots (mean [B2M, NGAL, NAG, IgG] or
median [RBP], confidence interval) for each analyte based on the year in which the urine sample was collected. Although all analytes except for NAG exhibited an increasing trend as storage time decreased, a statistically significant increase was not observed. The following $P$-values were obtained: $u_{\text{RBP/c}} = 0.072$; $u_{\text{B2M}} = 0.065$; $u_{\text{NGAL/c}} = 0.065$; $u_{\text{NAG/c}} = 0.624$; $u_{\text{IgG/c}} = 0.062$.

Table S1. Correlations (Corr) of urinary biomarkers in dogs with XLHN. For all correlations $P < .001$.

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