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Correlation of Urine and Serum Biomarkers with Renal Damage and Survival in Dogs with Naturally Occurring Proteinuric Chronic Kidney Disease

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Background: Urine protein loss is common in dogs with chronic kidney disease (CKD).

Hypothesis/Objectives: To evaluate new biomarkers of glomerular and tubulointerstitial (TI) damage compared with histology and as survival indicators in dogs with naturally occurring, proteinuric CKD.

Animals: One hunderd and eighty dogs with naturally occurring kidney disease.

Methods: Retrospective study using urine, serum, and renal biopsies from dogs with kidney disease, 91% of which had proteinuric CKD. Biomarkers were evaluated and correlated with pathologic renal damage, and significant associations, sensitivities, and specificities of biomarkers for renal disease type were determined.

Results: Fractional excretions of immunogloblin M (IgM_FE) and immunoglobulin G (IgG_FE) correlated most strongly with glomerular damage based on light microscopy (r = 0.58 and 0.56, respectively; $P < .01$). Serum creatinine (SCr) correlated most strongly with TI damage (r = 0.70, $P < .01$). Urine IgM/creatinine and urine NAG/creatinine had the highest sensitivity (75%) and specificity (78%) for detection of immune complex-mediated glomerulonephritis. Although individually most biomarkers were significantly associated with decreased survival time ($P < .05$), in a multivariate analysis, SCr, IgM_FE, and glomerular damage based on transmission electron microscopy (TEM) were the only biomarkers significantly associated with survival time (SCr: $P = .001$; IgM_FE: $P = .008$; TEM: $P = .017$).

Conclusions and Clinical Importance: Novel urine biomarkers and FEs are useful for detection of glomerular and TI damage in dogs with proteinuric CKD and might predict specific disease types and survival.

Key words: Immunoglobulin G; Immunoglobulin M; N-acetyl-β-D-glucosaminidase; Neutrophil gelatinase-associated lipocalin; Retinol binding protein.

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Abbreviations:

CKD	chronic kidney disease
FE	fractional excretion
HR	hazard ratio
ICGN	immune complex-mediated glomerulonephritis
IgG_FE	fractional excretion of immunoglobulin G
IgG	immunoglobulin G
IgM_FE	fractional excretion of immunoglobulin M
IgM	immunoglobulin M
IVRPS	International Veterinary Renal Pathology Service
LM	light microscopy
NAG	N-acetyl-β-D-glucosaminidase
NGAL_FE	fractional excretion of neutrophil gelatinase-associated lipocalin
NGAL	neutrophil gelatinase-associated lipocalin
O/E%	observed to expected ratio
RBP_FE	fractional excretion of retinol binding protein
RBP	retinol binding protein
SCr	serum creatinine
sIgG	serum immunoglobulin G
sIgM	serum immunoglobulin M
sNGAL	serum neutrophil gelatinase-associated lipocalin
TEM	transmission electron microscopy
uIgG/c	urine immunoglobulin G/creatinine
uIgM/c	urine immunoglobulin M/creatinine
uNAG/c	urine N-acetyl-β-D-glucosaminidase/creatinine
uNGAL/c	urine neutrophil gelatinase-associated lipocalin/creatinine
UPC	urine protein/creatinine
uRBP/c	urine retinol binding protein/creatinine
USG	urine specific gravity

Chronic kidney disease (CKD) is a common cause of morbidity and mortality in dogs,[1,2] and current noninvasive methods of diagnosis often lack sensitivity, specificity, or both for early disease detection and for identification of the underlying disease process. Clinically, CKD in dogs is typically detected by the presence of renal azotemia, persistent renal proteinuria, or both, often in conjunction with decreased urine concentrating ability, abnormal findings on urine sediment examination (such as presence of casts in the sediment), and abnormal appearance of the kidneys on ultrasound. Persistent renal proteinuria, typically quantified by measuring urine protein:creatinine (UPC), can be an early indicator of CKD in dogs,[1,3] and it is a negative prognostic factor in dogs with CKD.[4] However, when mildly increased, UPC cannot differentiate glomerular from tubular damage. Renal biopsy is considered the gold standard for determining the type of renal damage,[5] but it is an invasive procedure and is not feasible in every case because of financial constraints or animal health. Therefore, less invasive, inexpensive, sensitive and specific methods to evaluate the presence, character, and severity of kidney damage in dogs are needed.

Urine and serum biomarkers can be useful in human and veterinary medicine for early identification and localization of renal damage and as more sensitive and specific indicators of disease. In proteinuric kidney diseases, differently sized proteins are present in urine secondary to damage to different regions of the nephron (eg, glomeruli versus tubules). For example, the presence of high molecular weight proteins, such as immunoglobulins, in urine is indicative of glomerular damage.[6] By contrast, low molecular weight proteins and tubular enzymes are thought to be more specific for renal tubular damage.[6] Of these urine proteins, a few have been recently evaluated in veterinary medicine. Urine immunoglobulin G (uIgG) and urine retinol binding protein (uRBP) were increased in dogs with primary CKD[7] and those with renal dysfunction secondary to various systemic diseases including pyometra, babesiosis, and snake envenomation.[7–15] Urine, plasma, and serum neutrophil gelatinase-associated lipocalin (NGAL) and urine N-acetyl-β-D-glucosaminidase (uNAG), a renal tubular enzyme, are tubular markers increased in both acute and CKD in dogs.[7,11,12,15–22] Neutrophil gelatinase-associated lipocalin originates not only in the renal tubules but also from neutrophil granules and many other organs.[23] Novel urine biomarkers are not regularly used as diagnostic tools for evaluation of renal disease in veterinary medicine, and few veterinary studies correlate biomarkers with histologically proven renal damage[7,12,13,18] and case outcome.[4]

The objective of this study was to determine correlations of promising novel urine biomarkers of renal damage (IgG, immunoglobulin M (IgM), RBP, NGAL, and NAG) with pathologic assessment of glomerular and tubulointerstitial (TI) damage in dogs with naturally occurring, primarily proteinuric CKD attributable to a variety of causes. Our goal was to determine if the biomarkers provided an indication of the presence and severity of glomerular and/or TI damage, which would support their use as noninvasive tests to detect and monitor proteinuric CKD. We also determined sensitivities and specificities of the biomarkers for detection of specific types of renal disease and evaluated follow-up information from these dogs to determine if the biomarkers might be useful as survival indicators.

Materials and Methods

Sample Collection and Processing

This retrospective study used dog samples of urine supernatant, serum, and kidney tissue collected by the dog's veterinarian and submitted to the International Veterinary Renal Pathology Service (IVRPS) for diagnostic purposes between January 2008 and September 2013. All samples were shipped on ice and were typically received and processed the day following collection. Urine supernatant and serum were aliquoted and stored at −80°C until analysis. Cases were categorized as having inactive urine sediment, an active urinary tract infection (based on culture or sediment findings), hematuria [grossly or microscopically (>100 red blood cells per 40× field)], or pyuria (>10 white blood cells per 40× field), identified either on the submitted sample, if available, or within 4 weeks of renal biopsy. Cases with an active sediment were excluded from analysis. Renal biopsies were routinely processed for light (LM) and transmission electron microscopy (TEM) as previously described.[24] Criteria for diagnosis of renal disease included persistent proteinuria, azotemia, or both. Cases were categorized as having CKD, acute kidney injury (AKI), both CKD and AKI, or not enough information available to determine chronicity of renal disease. CKD was defined by evidence of renal disease for at least 3 months or evidence of chronicity on renal ultrasound or histology.

Histopathologic Analysis and Scoring

Renal biopsies were evaluated by a single pathologist (REC) for glomerular and TI damage. Glomerular damage was evaluated with LM and TEM, and TI damage was evaluated with LM. A variation in the scoring system developed for the World Small Animal Veterinary Association Renal Standardization Project[25] was used to indicate the amount and severity of glomerular and TI damage (Tables S1–S3). For TI damage, the final score consisted of an average of individual scores for each component of TI damage (interstitial fibrosis, tubular atrophy, degeneration/necrosis/regeneration, and interstitial chronic inflammation).

Assay Validation

Commercial assay kits for each biomarker (IgG,[a] IgM,[b] RBP,[c] NGAL,[d] NAG[e]) were used. The IgG, RBP, and NAG assays were previously validated using dog urine.[7] Assay validation for IgM and NGAL can be found in the supplemental materials and methods.

Biomarkers

All urine and serum biomarkers were analyzed in duplicate. Freeze-thaw cycles were limited to ≤5 per sample. Conventional biomarkers (serum creatinine (SCr),[f,h] urine protein:creatinine (UPC),[f,g,h] and urine specific gravity (USG)[i]) and novel biomarkers [urine and serum IgG (uIgG and sIgG), IgM (uIgM and sIgM), RBP (uRBP and sRBP), and NGAL (uNGAL and sNGAL) and urine NAG (uNAG)] were measured in our laboratory. Standards were optimized according to the manufacturer for

detection of RBP in dog samples. Urine biomarker concentrations were normalized to urine creatinine concentration (eg, uIgG/c). Using the spot sample approach,[26] fractional excretion (FE) of IgG, IgM, RBP, and NGAL (IgG_FE, IgM_FE, RBP_FE, and NGAL_FE) were calculated using the formula: $FE_{analyte} = (Analyte_{urine}/Analyte_{serum}) \times (SCr/Creatinine_{urine}) \times 100$. Cases were classified into CKD stages 1–4 based on the International Renal Interest Society (IRIS) guidelines (http://www.iris-kidney.com/guidelines/staging.shtml), realizing that some cases could represent acute or acute on chronic disease and therefore not be in steady state.

Survival Data

The referring veterinarian or owner for each dog was contacted from 6 months to 6 years post biopsy. If deceased, the following information was recorded: time to death post biopsy, whether death was spontaneous or because of euthanasia, and cause of death (renal-related or otherwise).

Statistical Analysis

Biomarker Correlations. Simple linear regression on standardized continuous variables was used to estimate the correlation between biomarkers and also between each biomarker and biopsy damage scores to determine which biomarker correlated best with renal injury. Standardization was performed by subtracting the mean of the variable from an individual result and dividing by the standard deviation of that variable. Simple linear regression was also used to estimate the correlation between glomerular and TI damage scores. Correlation strength was defined as follows: weak: r = 0.0–0.39; moderate: r = 0.4–0.69; strong: r = 0.7–1.0. The Kolmogorov–Smirnov test was used to assess normality of the residuals, and data were natural log or square root transformed as necessary. Simple linear regression modeling for groups was used to determine significant differences in biomarkers between IRIS stages.

Disease Type Prediction. Logistic regression was used to determine presence of significant associations between biomarkers and specific types of kidney diseases, based on histopathologic diagnosis, including immune complex-mediated glomerulonephritis (ICGN), glomerulosclerosis, amyloidosis, other nephropathies, and tubular disease. "Other nephropathies" included juvenile nephropathies (eg, maldevelopment) and nephropathies other than ICGN, glomerulosclerosis, amyloidosis, and primary tubular disease, such as cases with glomerular basement membrane and podocyte damage without immune complex deposition, cases with glomerular atrophy, and cases with primarily interstitial fibrosis. Receiver operator characteristic (ROC) analysis was performed to determine sensitivities and specificities for each biomarker with each disease type, and cutoff values for each biomarker were calculated based on that which maximized sensitivity and specificity. Sensitivities and specificities for disease types were also calculated for selected pairs of biomarkers. For each disease type, a dichotomous variable was created to be the response for the logistic model and ROC analysis (1 indicated the disease type of interest and 0 was otherwise).

Survival Analysis. For each biomarker and damage score, a survival model was fit, using a Cox semiparametric model (accounting for the biomarker/damage score and age as covariates) to estimate median time to death attributable to renal disease post biopsy using all follow-up data obtained (n = 98 dogs). Hazard ratios (HR) were used to describe the association of the biomarker and age with time to death. All data were also evaluated together in a multivariate Cox model to determine which combination of biomarkers and biopsy damage scores had the most significant association with time to death attributable to renal disease post biopsy as described by HR. For the Cox models, TEM glomerular damage scores were recategorized into "no damage to mild damage" (0 and 1) versus "moderate to severe damage" (2 and 3).

All statistical analyses were carried out using Stata version 13, setting $P < .05$.[j]

Results

Dogs/Samples

Urine supernatant, serum, and kidney tissue from 203 dogs were initially analyzed. Of these, 130 (64%) urine samples had urinalyses performed on the submitted sample by the referring veterinarian or on a sample within 4 weeks of biopsy collection. Twenty-three dogs had evidence of an ongoing or recent bacteriuria, pyuria, or hematuria, and these cases were completely excluded leaving 180 cases for further analysis.

Of the remaining 180 cases, there were 80 (44.4%) spayed females, 57 (31.7%) neutered males, 25 (13.9%) intact males, and 18 (10.0%) intact females.

Numerous breeds were represented; the most common breeds were: Labrador Retrievers/Labrador Retriever-mixes: 19 (10.6%); Golden Retrievers/Golden Retriever-mixes: 9 (5.0%); Yorkshire Terrier/Yorkshire Terrier-mixes: 9 (5.0%); Miniature Schnauzers: 7 (3.9%); Doberman Pinschers: 6 (3.3%); and Rottweiler/Rottweiler-mixes: 5 (2.8%).

The age range was 2 months to 14 years old, with a median of 7 years old. Ten dogs (5.6%) were 0 to <1 year; 45 (25.0%) were 1 year to <5 years; 101 (56.1%) were 5 to <10 years; and 22 (12.2%) were ≥10 years. Two dogs were of an unknown age.

Follow-up information was collected for 98 (54%) dogs; information regarding time from biopsy to death and cause of death was collected for 62 dogs, 51 of which died or were euthanized because of renal-related causes. Median time to death caused by renal disease post biopsy (excluding submitted necropsy samples) was 179 days (range: 2–1,349 days).

Kidney disease was diagnosed based on persistent proteinuria in 87 dogs (48.3%), azotemia in 19 dogs (10.6%), and both proteinuria and azotemia in 74 dogs (41.1%). CKD was confirmed for 165 (91.7%) dogs, while 3 dogs (1.7%) had concurrent CKD and AKI. Five dogs (2.8%) had AKI, and for 7 dogs (3.9%) chronicity of renal disease was unable to be determined.

Histopathologic Findings and Scores

Of 180 dogs included in the study, glomerular and TI damage were assessed in 176 dogs, whereas the remaining four did not have renal tissue available for evaluation. One hundred and fifty-one dogs had glomeruli available for evaluation by TEM, and the remaining 29 dogs did not have TEM performed for various reasons (eg, LM evaluation was sufficient for diagnosis, a TEM sample was not submitted, or glomeruli were not present in the TEM sample). Table 1 demonstrates that this study cohort overall had worse glomerular damage

Table 1. Percentage of cases in each category of glomerular damage biopsy scores based on LM and TEM and TI damage biopsy scores based on LM.

	Score 0–<1	Score 1–<2	Score 2–<3	Score ≥3
LM glomerular damage score n = 176	8.5%	26.7%	40.3%	24.4%
TEM glomerular damage score n = 151	2.0%	24.5%	43.7%	29.8%
TI damage score n = 176	58.5%	30.1%	10.8%	0.6%

LM, light microscopy; TEM, transmission electron microscopy; TI, tubulointerstitial.

than TI damage. Cases were divided into 5 disease categories with the following distribution: ICGN: 62 (34.4%); glomerulosclerosis: 47 (26.1%); amyloidosis: 18 (10.0%); other nephropathies: 32 (17.8%); and primary tubular disease: 15 (8.3%). Biopsies from 6 (3.3%) dogs were not assigned a disease category because the biopsied regions were either normal or insufficient to make a complete disease diagnosis; these cases were removed from the disease type prediction analysis. However, for 4 of these 6 cases, there was adequate tissue for either glomerular evaluation (n = 2), or TI evaluation (n = 2). Correlation between TI and LM glomerular damage scores was moderate (r = 0.45, $P < .001$), whereas there was no correlation between TI and TEM glomerular damage scores (r = −0.03).

Assay Validation

Analytical performance of the NGAL and IgM assays was acceptable (Table S4).

Biomarker Findings

On average, dogs were mildly to moderately azotemic (107 dogs (59.4%) had SCr ≥ 1.4 mg/dL) and moderately to markedly proteinuric (137 dogs (76.1%) had UPC ≥ 2.0) (Table 2). Twenty-seven (15%) dogs had a UPC 0.5–2.0 and 16 (9%) had UPC < 0.5. Of these 43 dogs with UPC < 2.0, 23.3% had primary glomerular disease as determined by histopathology. Of the dogs with UPC < 0.5 (n = 16), 1 dog (6.3%) had primary glomerular disease.

Biomarkers demonstrated a large range of values, and typically urine biomarkers were higher than have previously been reported in clinically healthy dogs.[7,15–17,21,27] For 66.6% of the 180 cases included in the study, all biomarkers were measured; the remaining 33.4% of cases did not have a complete biomarker set. Urine immunoglobulin G/creatinine, uIgM/c, and uRBP/c were measured for 100% of the cases. Serum immunoglobulin G, sIgM, and sRBP were measured for all cases which had submitted serum samples (76.1% of cases). Serum neutrophil gelatinase-associated lipocalin and uNGAL/c were measured for 68.9% and 85% of cases, respectively. Urine N-acetyl-β-D-glucosaminidase/creatinine was measured for 94.4% of cases. When only cases that had a complete set of biomarker data were included in the statistical analyses, results of each analysis were similar to results when all cases (ie, those with and without a complete biomarker set) were included (data not shown). While FE for most biomarkers was <100%, NGAL_FE ranged from 0 to 506%. With regard to IRIS stages, only RBP_FE and NGAL_FE demonstrated significantly progressive increases with higher IRIS stages, although all novel biomarkers except uIgG/c and uNAG/c tended to increase with higher stages of disease (Table S5).

Biomarker Correlations. Of 45 combinations of urine protein biomarkers (normalized urine concentration or FE), 82.2% showed moderate to strong correlations with each other and with UPC (Table S6). By contrast, serum protein biomarker concentrations (sRBP and sNGAL) generally demonstrated weak correlations with other biomarkers, and the highest correlation was observed with the urine concentration of the same biomarker (eg, sRBP with uRBP/c). SCr correlated only weakly to moderately with FE of the biomarkers, with the strongest (but still moderate) correlation for SCr being with RBP_FE.

Glomerular damage based on LM correlated best (albeit moderately) with FE of high molecular weight (ie, "glomerular") biomarkers (IgM_FE: r = 0.58; IgG_FE: r = 0.56), and both IgM_FE and IgG_FE had stronger correlations with glomerular damage than did UPC (r = 0.45) (Fig 1). The remaining urine protein biomarkers (urine concentrations and FEs) correlated less strongly with glomerular damage (range: r = 0.32–0.47). Correlations of many biomarkers with glomerular damage were stronger when based on TEM compared with LM (Fig 2).

For TI damage, SCr had the strongest correlation (r = 0.7, Fig 3). Retinol binding protein was the only biomarker where all measurements (urine and serum) significantly correlated with TI damage, with RBP_FE demonstrating the strongest, albeit moderate, correlation (r = 0.58). The only other significant correlations with TI damage were FEs of the other biomarkers as well as USG, which demonstrated a weak, negative correlation.

Disease Type Prediction. We used logistic regression to determine if any of the biomarkers was significantly associated with each diagnostic category (Table 2). ROC analysis and sensitivity and specificity calculations for each disease type were also performed for each biomarker. Individually, increased uIgM/c, uIgG/c, uNAG/c, and UPC were significantly associated with ICGN based on logistic regression, and these demonstrated the highest sensitivities and specificities for ICGN. A ROC analysis of pairs of biomarkers revealed uIgM/c and uNAG/c to be the only combination of biomarkers significantly associated with ICGN. This combination, using cutoff values of uIgM/c > 7.3 μg/mg and uNAG/c > 7.0 U/g, had a sensitivity and specificity for detection of ICGN of 75% and 78%, respectively, which was similar to that of uIgM/c alone. Individually,

Table 2. Median (range) of biomarker values in dogs with naturally occurring chronic kidney disease. For biomarkers that were significantly associated with specific categories of disease according to logistic regression, optimal cutoff values, and corresponding sensitivities and specificities, as determined by receiver operator characteristic (ROC) analysis, are displayed.

	All Dogs		ICGN		Glomerulosclerosis		Amyloidosis		Other Nephropathies		Tubular Disease	
	n	Median (Range)	n	Median (Range) Cutoff (Se%, Sp%)	n	Median (Range) Cutoff (Se%, Sp%)	n	Median (Range) Cutoff (Se%, Sp%)	n	Median (Range) Cutoff (Se%, Sp%)	n	Median (Range) Cutoff (Se%, Sp%)
SCr (mg/dL)	179	1.5 (0.3–21.8)	61	1.5 (0.3–9.3)	47	1.4 (0.3–12.0)	18	1.1 (0.5–4.9)	32	1.7 (0.6–21.8)* >2.5 (37.5, 78.2)	15	2.1 (0.5–13.0)* >3.2 (46.7, 87.2)
USG	180	1.017 (1.003–1.048)	62	1.019 (1.005–1.046)** >1.014 (80.6, 47.5)	47	1.017 (1.003–1.048)	18	1.013 (1.003–1.027)* <1.013 (50.0, 73.5)	32	1.014 (1.005–1.047)	15	1.013 (1.005–1.026)* <1.014 (60.0, 69.1)
UPC Ratio	180	5.0 (0.0–36.8)	62	8.6 (0.4–36.8)** >2.5 (95.2, 40.7)	47	5.2 (0.5–31.6)	18	11.0 (1.7–21.3)	32	1.5 (0.0–14.3)** <2.5 (71.9, 81.8)	15	0.5 (0.0–6.0)** <2.0 (86.7, 81.2)
IgG												
uIgG/c (μg/mg)	180	554.0 (0.6–37,649.0)	62	1,212 (27–37,649)** >729.0 (62.9, 72.9)	47	562.5 (48.7–7,070.3)	18	727.0 (128.9–6,589.0)	32	104.1 (0.6–1,938.5)** <374.7 (87.5, 71.6)	15	37.3 (0.7–489.3)** <211.0 (86.7, 80.0)
IgG_FE%	137	0.1 (0.0–8.6)	48	0.1 (0.0–8.6)	36	0.1 (0.0–1.4)	12	0.2 (0.0–3.2)	27	0.0 (0.0–4.7)	10	0.0 (0.0–0.2)
IgM												
uIgM/c (μg/mg)	180	6.4 (0.3–557.0)	62	14.6 (2.1–557.0)** >8.0 (74.2, 75.4)	47	5.6 (0.5–26.9)* <13.5 (87.2, 32.3)	18	5.7 (3.1–24.3)	32	3.1 (0.4–20.2)** <5.6 (75.0, 60.1)	15	2.0 (0.3–28.3)* <3.5 (73.3, 74.5)
IgM_FE%	137	0.003 (0.000–0.172)	48	0.008 (0.000–0.145)* >0.005 (62.5, 76.4)	36	0.002 (0.000–0.022)* <0.005 (83.3, 45.5)	12	0.002 (0.001–0.019)	27	0.002 (0.000–0.172)	10	0.001 (0.000–0.033)
RBP												
uRBP/c (μg/mg)	180	8.7 (0.0–1,013.4)	62	9.3 (0.1–243.8)	47	10.0 (0.0–1,013.4)	18	2.1 (0.0–78.4)	32	4.0 (0.0–402.9)	15	1.6 (0.0–306.1)
sRBP (μg/mL)	137	105.5 (22.9–414.2)	48	90.1 (22.9–280.5)* <112.8 (70.8, 55.1)	36	139.6 (30.9–258.2)** >112.2 (75.0, 64.4)	12	80.9 (44.2–126.0)	27	109.2 (26.7–414 .2)	10	96.2 (39.3–160.1)
RBP_FE%	137	0.1 (0.0–24.9)	48	0.2 (0.0–18.0)	36	0.1 (0.0–2.7)	12	0.1 (0.0–3.2)	27	0.0 (0.0–14.3)	10	0.0 (0.0–24.9)* >1.5 (30.0, 89.8)
NGAL												
uNGAL/c (ng/mg)	153	93.6 (0.0–1,533.4)	53	107.5 (8.2–1,533.4)	41	102.6 (9.7–842.1)	13	92.2 (13.1–321.7)	29	63.1 (0.0–660.6)	11	43.5 (4.2–496.9)
sNGAL (ng/mL)	124	12.1 (2.4–149.1)	44	14.0 (2.6–65.7)	34	13.1 (3.3–149.1)	10	11.0 (4.8–23.4)	24	8.9 (2.4–106.8)	7	8.3 (2.8–24.7)
NGAL_FE%	124	10.8 (0.0–505.9)	44	11.7 (0.5–82.8)	34	10.1 (1.9–86.1)	10	13.3 (2.2–40.4)	24	7.5 (0.0–505.9)	7	3.6 (0.2–26.8)
uNAG/c (U/g)	170	13.5 (0.4–427.7)	57	26.5 (3.0–427.7)** >23.8 (56.1, 85.0)	46	12.7 (2.0–76.7)	18	20.4 (3.3–67.2)	30	7.4 (0.6–37.7)** <9.4 (63.3, 71.4)	13	2.8 (0.4–10.3)** <6.9 (92.3, 79.6)

ICGN, immune complex-mediated glomerulonephritis; Se%, sensitivity; Sp%, specificity; SCr, serum creatinine; USG, urine specific gravity; UPC, urine protein:creatinine ratio; uIgG/c, urine immunoglobulin G/urine creatinine; IgG_FE, fractional excretion of immunoglobulin G; uIgM/c, urine immunoglobulin M/urine creatinine; IgM_FE, fractional excretion of immunoglobulin M; uRBP/c, urine retinol binding protein/urine creatinine; sRBP, serum retinol binding protein; RBP_FE, fractional excretion of retinol binding protein; uNGAL/c, urine neutrophil gelatinase-associated lipocalin/urine creatinine; sNGAL, serum neutrophil gelatinase-associated lipocalin; NGAL_FE, fractional excretion of neutrophil gelatinase-associated lipocalin; uNAG/c, urine N-acetyl-β-D-glucosaminidase/urine creatinine.

Biomarker is significantly associated with type of kidney disease according to logistic regression: *$P < .05$, **$P < .01$; Outcome for ROC analysis in determining sensitivity and specificity was presence of a particular disease. Cutoff values were determined by those which maximize the area under the ROC curve, and greater or less than the cutoff value was determined by the increased probability of having the disease when above or below the cutoff value.

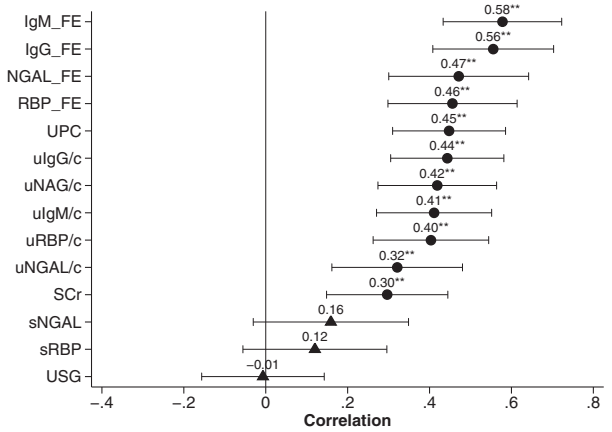

Fig 1. Correlations of biomarkers with glomerular damage based on light microscopy, with 95% confidence intervals. Circles represent a statistically significant correlation and triangles represent no significant correlation. $^{**}P < .01$; n = 176. SCr, serum creatinine; USG, urine specific gravity; UPC, urine protein: creatinine ratio; uIgG/c, urine immunoglobulin G/urine creatinine; IgG_FE, fractional excretion of immunoglobulin G; uIgM/c, urine immunoglobulin M/urine creatinine; IgM_FE, fractional excretion of immunoglobulin M; uRBP/c, urine retinol binding protein/urine creatinine; sRBP, serum retinol binding protein; RBP_FE, fractional excretion of retinol binding protein; uNGAL/c, urine neutrophil gelatinase-associated lipocalin/urine creatinine; sNGAL, serum neutrophil gelatinase-associated lipocalin; NGAL_FE, fractional excretion of neutrophil gelatinase-associated lipocalin; uNAG/c, urine N-acetyl-β-D-glucosaminidase/urine creatinine.

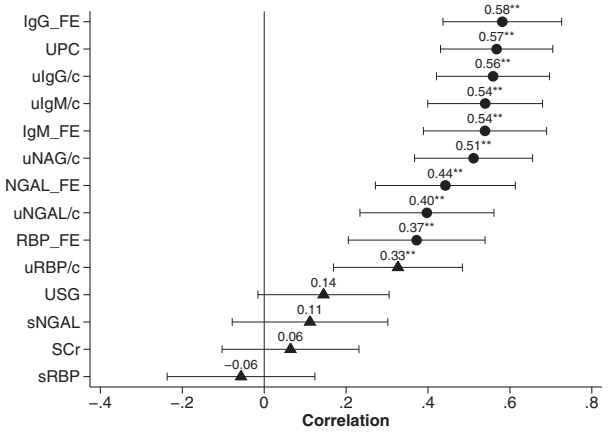

Fig 2. Correlations of biomarkers with glomerular damage based on transmission electron microscopy with 95% confidence intervals. Circles represent a statistically significant correlation and triangles represent no significant correlation. $^{**}P < .01$; n = 151. SCr, serum creatinine; USG, urine specific gravity; UPC, urine protein:creatinine ratio; uIgG/c, urine immunoglobulin G/urine creatinine; IgG_FE, fractional excretion of immunoglobulin G; uIgM/c, urine immunoglobulin M/urine creatinine; IgM_FE, fractional excretion of immunoglobulin M; uRBP/c, urine retinol binding protein/urine creatinine; sRBP, serum retinol binding protein; RBP_FE, fractional excretion of retinol binding protein; uNGAL/c, urine neutrophil gelatinase-associated lipocalin/urine creatinine; sNGAL, serum neutrophil gelatinase-associated lipocalin; NGAL_FE, fractional excretion of neutrophil gelatinase-associated lipocalin; uNAG/c, urine N-acetyl-β-D-glucosaminidase/urine creatinine.

low UPC, uIgG/c, and uNAG/c were most significantly associated with primary tubular disease; however, ROC analysis did not reveal a combination of biomarkers that was significantly associated with primary tubular disease.

Survival Analysis. In this cohort of dogs with primarily proteinuric CKD, increases in SCr, IgM_FE, uRBP/c, RBP_FE, NGAL_FE, and IgG_FE, as well as TI, LM, and TEM glomerular damage scores were all significantly associated with shortened time to death due to renal disease according to Cox survival models including age as a covariate (Table 3). For example, an increase in SCr of 1 mg/dL resulted in an increased hazard of death of 40%, while an increase of 0.01% for IgM_FE resulted in an increased hazard of death of 45%. An increase in TI damage score or LM glomerular damage score of 1 point (eg, an increase from 0 to 1 or from 1 to 2) resulted in increased hazards of death of 160% and 60%, respectively, while an increase in TEM glomerular damage score from 0/1 to 2/3 resulted in an increased hazard of death of 158%. Age also had a significant association with time to death when combined in the survival models for IgM_FE, RBP_FE, and IgG_FE (ie, a 1-year increase in age increased the hazard of death associated with these biomarkers). Age did not have a significant association with time to death when combined in survival models for SCr, uRBP/c, NGAL_FE, TI damage score, and LM or TEM glomerular damage scores.

To determine which combination of biomarkers and damage scores was significantly associated with time to death due to renal disease in this cohort of dogs, all biomarkers were evaluated together in a multivariate Cox survival model. Notably, SCr, IgM_FE, and TEM glomerular damage scores were the only parameters that were significantly associated with time to death due to renal disease post biopsy (Table 4). Survival graphs for these 3 variables demonstrate the probability of survival for a dog of median age (7 years) based on different starting levels of each biomarker or damage score (Fig 4). Survival graphs also demonstrate the probability of survival for dogs with varying combinations of SCr and IgM_FE values based on a TEM glomerular damage score of either 0/1 or 2/3 (Fig S1).

Discussion

This study evaluated 5 novel biomarkers in 180 dogs with naturally occurring kidney disease, primarily proteinuric CKD typically caused by primary glomerular disease. Several of these urine biomarkers and their FEs correlated with pathologic severity of glomerular damage, TI damage, or both. Increased uIgM/c and uNAG/c were most significantly associated with ICGN. Furthermore, increased SCr, IgM_FE, uRBP/c, RBP_FE, NGAL_FE, and IgG_FE, as well as TI damage scores and LM and TEM glomerular damage scores were associated with reduced survival when variables were

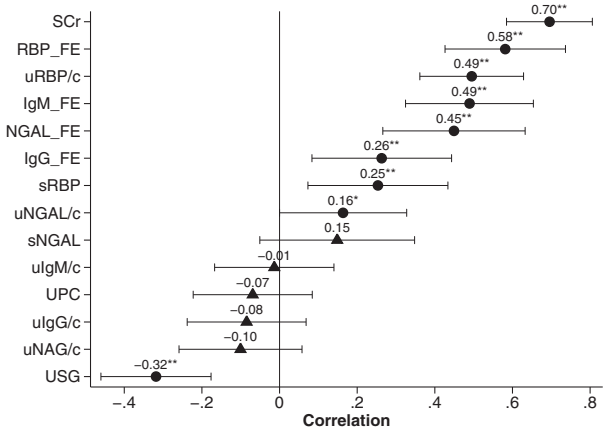

Fig 3. Correlations of biomarkers with tubulointerstitial damage based on light microscopy with 95% confidence intervals. Circles represent a statistically significant correlation and triangles represent no significant correlation. $*P < .05$; $**P < .01$; n = 176. SCr, serum creatinine; USG, urine specific gravity; UPC, urine protein: creatinine ratio; uIgG/c, urine immunoglobulin G/urine creatinine; IgG_FE, fractional excretion of immunoglobulin G; uIgM/c, urine immunoglobulin M/urine creatinine; IgM_FE, fractional excretion of immunoglobulin M; uRBP/c, urine retinol binding protein/urine creatinine; sRBP, serum retinol binding protein; RBP_FE, fractional excretion of retinol binding protein; uNGAL/c, urine neutrophil gelatinase-associated lipocalin/urine creatinine; sNGAL, serum neutrophil gelatinase-associated lipocalin; NGAL_FE, fractional excretion of neutrophil gelatinase-associated lipocalin; uNAG/c, urine N-acetyl-β-D-glucosaminidase/urine creatinine.

modeled separately. However, in a multivariate model, only SCr, IgM_FE, and TEM glomerular damage scores were associated with time to death attributable to renal-related causes. Our findings support that conventional markers of kidney disease (SCr, UPC) correlate with kidney damage either similarly to or better than the novel biomarkers evaluated in this study. However, novel biomarkers can provide useful additional information to support the presence of glomerular or tubular damage, to help distinguish between disease categories, and to inform prognosis.

This study did not reveal superiority of any novel biomarker to SCr with regard to TI damage or survival. However, SCr is often not optimally interpreted, with diagnosis of azotemia based on exceeding a reference interval rather than what is "normal" or baseline for a particular animal. This is especially problematic for small breed dogs and animals with concurrent muscle wasting, the latter of which is a common finding in CKD and will complicate monitoring for disease progression.[28] Therefore, having additional noninvasive markers of TI damage and dysfunction would be useful to confirm SCr interpretations. In addition, even though SCr demonstrated the best correlation with TI damage, the majority (59%) of dogs evaluated in this study was already at least mildly azotemic at the time of biopsy, and it is possible that evaluation of urine biomarkers might help identify TI damage earlier than SCr.

Fractional excretions of IgG and IgM correlated slightly stronger than UPC with severity of glomerular damage when evaluated with LM; however, when glomerular damage was assessed with TEM, IgG_FE, UPC, and uIgG/c provided the best indications of ultrastructural glomerular damage. Thus, UPC is likely an effective conventional marker of ultrastructural glomerular damage. Of interest is the use of uIgM/c, uIgG/c, and uNAG/c, particularly the combination of uIgM/c and uNAG/c, for the identification of ICGN

Table 3. Association of biomarker/damage score and age with time to death caused by renal disease in dogs from multivariate Cox survival models. The unit increase for each biomarker/damage score and age is depicted in parentheses in the first column. Each row depicts a separate survival model that includes 2 covariables (biomarker or damage score and age).

		HR for Biomarker/Damage Score		HR for Age	
	N	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Biomarker + Age (1 year)					
SCr (1 mg/dL)	82	1.40 (1.26–1.56)	<.001	1.10 (0.99–1.22)	.072
IgM_FE (0.01%)	66	1.45 (1.25–1.69)	<.001	1.15 (1.00–1.32)	.047
uRBP/c (10 μg/mg)	83	1.07 (1.03–1.10)	<.001	1.07 (0.98–1.18)	.14
RBP_FE (1%)	66	1.17 (1.07–1.28)	.001	1.16 (1.01–1.33)	.034
NGAL_FE (25%)	59	1.23 (1.07–1.41)	.003	1.09 (0.95–1.25)	.20
IgG_FE (1%)	66	1.47 (1.11–1.95)	.007	1.15 (1.00–1.31)	.044
Damage score + Age (1 year)					
TI damage score (1 score point)	82	2.60 (1.59–4.24)	<.001	1.07 (0.97–1.17)	.17
LM glomerular damage score (1 score point)	82	1.60 (1.12–2.29)	.009	1.08 (0.98–1.20)	.13
TEM glomerular damage score (0/1 versus 2/3)	72	2.58 (1.03–6.44)	.042	1.05 (0.94–1.18)	.37

HR, hazard ratio; CI, confidence interval; LM, light microscopy; TEM, transmission electron microscopy; TI, tubulointerstitial; SCr, serum creatinine; IgG_FE, fractional excretion of immunoglobulin G; IgM_FE, fractional excretion of immunoglobulin M; uRBP/c, urine retinol binding protein/urine creatinine; RBP_FE, fractional excretion of retinol binding protein; NGAL_FE, fractional excretion of neutrophil gelatinase-associated lipocalin.

Table 4. Association of biomarkers and TEM glomerular damage score with time to death caused by renal disease in dogs from a multivariate Cox survival model (n = 84). The unit increase for each biomarker/damage score is depicted in parentheses in the first column.

Biomarker/Damage Score	HR (95% CI)	*P* Value
SCr (1 mg/dL)	1.40 (1.15–1.70)	.001
IgM_FE (0.01%)	1.28 (1.07–1.55)	.008
TEM glomerular damage score (0/1 versus 2/3)	4.80 (1.32–17.50)	.017

SCr, serum creatinine; IgM_FE, fractional excretion of immunoglobulin M; TEM, transmission electron microscopy; HR, hazard ratio; CI, confidence interval.

(discussed further below). Increased IgM_FE was also associated with a significantly increased hazard of death. This is similar to studies in humans, where increased urine IgM excretion in diabetic glomerulonephropathy was shown to be associated with increased risk of renal failure and death.[29,30] Because IgM is a large protein (~900 kDa), its presence in the urine might reflect more severe and possibly irreversible damage to the glomerular filtration barrier. In contrast to a previous study in dogs,[4] UPC was not associated with survival time. This could be because this previous study included dogs with SCr ranging from 2.0 to 8.0 mg/dL,[4] whereas many of the dogs in this study were not azotemic, and some had reversible glomerular injury. Treatment to reduce proteinuria both before and after renal biopsy might have also influenced results in this study.

The tubular markers SCr, RBP_FE, uRBP/c, and NGAL_FE typically correlated more strongly with TI than glomerular damage. However, all but SCr also correlated moderately well with glomerular damage. Similarly, IgM_FE correlated moderately with TI damage, although a stronger correlation was observed with glomerular damage. This is similar to a study of urine biomarkers in dogs with X-linked hereditary nephropathy, where SCr, uRBP/c, uNGAL/c, uNAG/c, and uIgG/c all correlated moderately to strongly with both glomerular and tubular lesions.[7] One possible explanation is that concurrent glomerular and tubular damage commonly occurs in dogs with CKD, and certainly, damage to 1 compartment will affect the other.

The use of TEM in this study identified biomarkers that were better for differentiating glomerular from tubular disease, including UPC, uIgG/c, uIgM/c, uNAG/c, and IgG_FE. Notably, most "glomerular" biomarkers had a stronger correlation with the TEM assessment of glomerular damage compared to LM evaluation, suggesting that TEM is better for determining the severity of glomerular filtration barrier damage. Furthermore, glomerular damage based on TEM, but not LM, was significantly associated with survival time and therefore may be more predictive of prognosis. However, both LM and TEM are needed for the comprehensive assessment of kidney biopsies. Light microscopy allows for evaluation of many glomeruli, which is particularly important for identification of scattered sclerotic or obsolescent glomeruli. Transmission electron microscope provides a more detailed structural view of the glomerulus, particularly the glomerular filtration barrier,

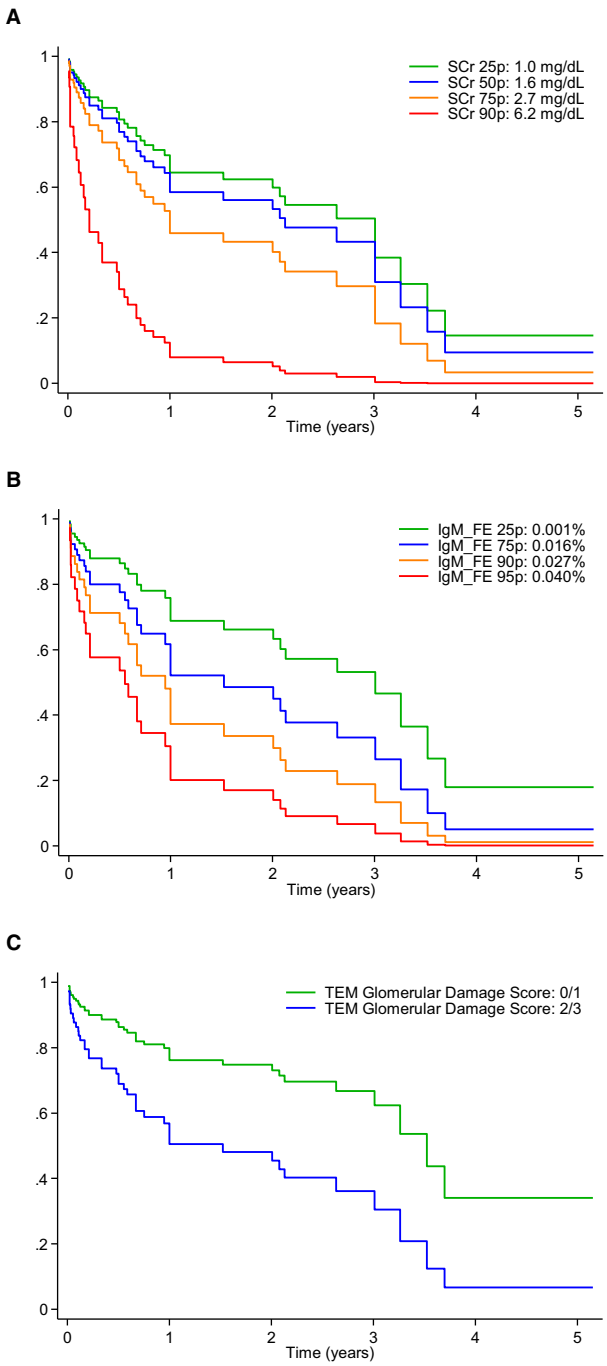

Fig 4. Probability of survival by biomarker/damage score for a dog at median age (7 years) at different starting values of biomarkers/damage scores for (**A**) SCr (n = 83); (**B**) IgM_FE (n = 67); (**C**) TEM Glomerular Damage Score (n = 73). 25p: 25th percentile; 50p: 50th percentile; 75p: 75th percentile; 90p: 90th percentile; 95p: 95th percentile. SCr, serum creatinine; IgM_FE, fractional excretion of immunoglobulin M; TEM, transmission electron microscopy.

but is not routinely performed outside a specialized biopsy service. In addition, it is usually performed on small tissue samples in which glomeruli may be absent.

Interestingly, uNAG/c correlated as strongly with glomerular damage as the glomerular markers but did not significantly correlate with TI damage. N-acetyl-β-D-glucosaminidase is a tubular lysosomal enzyme recognized as a marker of tubular injury, wherein tubular damage causes release of NAG and subsequent increases in enzyme activity in urine.[31,32] N-acetyl-β-D-glucosaminidase, which is approximately the size of IgG, does not pass through a normal glomerular filtration barrier, and the upper reference limit for uNAG/c in healthy dogs (3.63 U/g)[27] is well below the mean in this study. Previous studies have shown increased uNAG/c in dogs with CKD, presumed to be because of tubular damage or increased lysosomal turnover secondary to proteinuria.[7,15,19] While it is still possible that NAG leakage is occurring without histologic evidence of tubular damage, the strong correlation with glomerular damage and lack of correlation with TI damage in this study supports the possibility that NAG can pass through an injured glomerular filtration barrier. Therefore, while uNAG/c has been used to detect tubular damage in cases of acute kidney injury, it might also be useful to detect glomerular damage in chronic proteinuric nephropathies.

Another unexpected finding was the similar correlation of IgM_FE with both TI and glomerular damage. This is particularly intriguing given that uIgM/c did not correlate with TI damage. Fractional excretions of RBP, NGAL, and IgG also correlated more strongly with TI damage than their urine concentrations. This suggests that determination of FE could be more valuable than urine concentration of these markers for assessment of tubular damage, possibly because of the decreased ability of damaged tubular epithelial cells to reabsorb these proteins.

Maximum FEs observed for most biomarkers were <100%, except for NGAL_FE which reached 506%. This could indicate a large amount of secretion or loss of NGAL from damaged tubular cells. Alternatively, pyuria might have been present in samples that did not have a concurrent sediment examination despite recent results indicating inactive urine sediments. However, NGAL_FE in samples with known pyuria only reached up to 227% (data not shown).

An intriguing aspect of this study is the possibility that certain biomarkers might be able to predict specific disease types. In particular, markedly increased uIgM/c, uNAG/c, uIgG/c, and UPC were significantly associated with ICGN, which may be because of immune deposits creating large "holes" within the glomerular filtration barrier. A combination of uIgM/c and uNAG/c had a sensitivity and specificity for ICGN of 75% and 78%, respectively. While these values are not considerably high, they demonstrate promise in our ability to detect ICGN without a renal biopsy. However, further studies with larger populations of dogs are needed. While low UPC, uIgG/c, and uNAG/c were significantly associated with primary tubular disease on individual analysis, bivariate analysis did not reveal a combination of biomarkers with improved sensitivity or specificity over the individual analyses. It is possible that this might be because of an insufficient number of cases with primary tubular disease.

It was expected that TI damage score and SCr would be significantly associated with prognosis since TI damage is most closely associated with clinical parameters (glomerular filtration rate and SCr).[33] However, many parameters (uRBP/c, RBP_FE, IgM_FE, IgG_FE, and NGAL_FE, and LM and TEM glomerular damage scores) were also significantly associated with survival time. Since uRBP/c was shown to increase earlier than SCr,[7] increases in urinary biomarkers might provide an earlier indication of prognosis in dogs with renal disease. In a multivariate survival model, SCr was the most informative biomarker. However, IgM_FE and TEM glomerular damage scores were also significantly associated with survival time, and IgM_FE was more strongly associated than TEM findings. This suggests that the combination of SCr and IgM_FE might better predict prognosis than biopsy findings, although renal biopsy remains the gold standard for diagnosis and guide to therapy. Even very small changes in IgM_FE predicted significant changes in prognosis. This could be because IgM is a very large protein, likely requiring more severe damage to the glomerulus in order to pass into the urine filtrate. TI damage score, on the other hand, was not significantly associated with survival time in the multivariate analysis, possibly because many of the cases in this study were biopsied for suspected nonazotemic glomerular disease and therefore had low TI damage scores. Finally, increasing age was irrelevant to survival time once biomarker values and damage scores were known. Given that these survival predictions are based primarily on a population of dogs with proteinuric CKD, typically caused by glomerular disease, these biomarkers may not hold the same prognostic value in dogs with other etiologies of kidney disease.

Because most of the dogs in this study had proteinuric CKD, there was a bias for dogs with glomerular disease, which is a limitation of this study. Even so, 43 dogs (24%) had UPC < 2, which is not typically considered indicative of primary glomerular disease, and 16 of these dogs (9% of the entire cohort) had a UPC < 0.5. Of the 43 dogs with UPC < 2, 23% had histologic evidence of primary glomerular disease. Of those with UPC < 0.5 (n = 16), only 1 dog (6%) had primary glomerular disease. The distribution of the inciting cause of CKD in the general canine population is currently unknown, as it has not been comprehensively studied using clinicopathologic data, LM, and TEM; however, glomerular damage and proteinuria is common in dogs with CKD. While this study cohort does not completely represent the general population of dogs with CKD, a wide variety of naturally occurring kidney diseases were included.

A second limitation of the study is that not all urine samples had a corresponding urinalysis. The majority (64%) of the samples had a corresponding urinalysis performed either on the urine sample submitted with

the biopsy or within 4 weeks of the biopsy. Most samples were collected by internists at referral centers who performed a complete medical evaluation, minimizing the likelihood that significant sediment abnormalities were present when urinalysis results were not provided. However, studies have demonstrated that urinary tract infections and hematuria might alter biomarker levels,[34] and presence of infection could potentially increase systemic immunoglobulins. Therefore, all cases with known or suspected pyuria, bacteriuria, and/or marked hematuria were excluded from analyses to avoid interpreting increases in biomarkers that might be because of infection or hematuria.

Additional limitations include the unknown stability of the urine biomarkers, the variable time between collection and processing (although typically just 1 day), and the variability in length of sample storage before biomarker determination. The use of spot samples for calculation of FE, while an accepted method, is considered less accurate than the clearance approach for calculation of FE.[26] However, the spot sample approach is more feasible in clinical practice. In addition, not all biomarkers were measured in each dog; however, this was unlikely to have skewed the data as results were similar even if analysis was performed only for those cases with a complete biomarker set.

In conclusion, use of conventional biomarkers that are currently available for the diagnosis and monitoring of kidney disease, particularly SCr and UPC, are reasonable for assessment of kidney disease if used appropriately. A number of novel biomarkers are useful to detect glomerular or TI damage and potentially predict specific disease types and survival in dogs with naturally occurring CKD. In addition, analysis of quantitative pathologic biopsy scores, as based on the recently published World Small Animal Veterinary Association manuscript[25], can aid in prognostication in dogs with CKD. More studies are needed using a larger cohort of dogs to determine if specific biomarkers such as uIgM/c and uNAG/c can help in noninvasive diagnosis of ICGN in dogs. Furthermore, although uNAG/c has been reported as a marker of TI damage, it might be better suited as a marker of glomerular damage in dogs with proteinuric nephropathies.

Footnotes

[a] Dog IgG ELISA Quantitation Set, Bethyl Laboratories Inc., Montgomery, TX
[b] Dog IgM ELISA Quantitation Set, Bethyl Laboratories Inc., Montgomery, TX
[c] Human Retinol Binding Protein ELISA Kit, Immunology Consultants Laboratory, Inc., Newberg, OR
[d] Dog NGAL ELISA Kit, Immunology Consultants Laboratory, Inc., Newberg, OR
[e] N-acetyl-β-D-glucosaminidase (NAG) Assay, Diazyme Laboratories, Poway, CA
[f] Creatinine LiquiColor Test (Endpoint), Stanbio Laboratory, Boerne, TX
[h] Protein, Micro LiquiColor Test (CSF and Urine), Stanbio Laboratory, Boerne, TX
[g] Sirrus Clinical Chemistry Analyzer, Stanbio Laboratory, Boerne, TX
[i] Rhino VET360 Veterinary Clinical Refractometer, Reichert Technologies, Depew, NY
[j] Stata Corp. LP, College Station, TX

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Conflict of Interest Declaration: Authors disclose no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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Supporting Information

Additional Supporting Information may be found online in Supporting Information:

Data S1. Materials and methods.

Table S1. Glomerular scoring system based on light microscopy.

Table S2. Glomerular scoring system based on transmission electron microscopy.

Table S3. Tubulointerstitial scoring system based on light microscopy.

Table S4. Results from IgM and NGAL assay validation including mean inter- and intra-assay variability for low, middle, and high concentration urine samples, dilutional linearity, and spiking recovery.

Table S5. Median (range) for biomarkers within each IRIS stage.

Table S6. Correlation among biomarkers for dogs with naturally occurring chronic kidney disease.

Fig S1. Probability of survival for dogs with combinations of SCr and IgM_FE at the 25th percentile (1.0 mg/dL and 0.001%, respectively) and 95th percentile (5.0 mg/dL and 0.075%, respectively) based on transmission electron microscopy (TEM) scores: (A) TEM score 0/1 and (B) TEM score 2/3 (n = 60).