Neutrophil Gelatinase-Associated Lipocalin in Cats with Naturally Occurring Chronic Kidney Disease

I.-C. Wang, W.-L. Hsu, P.-H. Wu, H.-Y. Yin, H.-J. Tsai, and Y.-J. Lee

Background: Neutrophil gelatinase-associated lipocalin (NGAL) is a biomarker for the early prediction of renal damage and the progression of chronic kidney disease (CKD) in humans and dogs.

Hypothesis: Neutrophil gelatinase-associated lipocalin also may play a role in the progression of CKD in cats.

Animals: Eighty CKD and 18 control cats.

Methods: Cats were categorized into different stages according to the International Renal Interest Society (IRIS) staging system. Urine and plasma samples were collected and tested for NGAL concentrations using an in-house sandwich ELISA system and urinary NGAL (uNGAL)-to-creatinine ratio (UNCR) was determined. Cats in which serum creatinine concentration increased by >0.5 mg/dL from baseline within 30 days were defined as exhibiting progression.

Results: The urinary NGAL and UNCR of CKD cats were significantly higher than those of healthy cats ($P < .05$) and were highly correlated with serum creatinine concentration. The area under the receiver operating characteristic curve (AUROC) for uNGAL, when predicting the progression of CKD, was 0.71 and the best cutoff value was 2.06 ng/mL with a sensitivity of 76.9% and a specificity of 74.3%. The AUROC for UNCR when predicting the progression of CKD was 0.79 and the best cutoff value was 4.08 × 10$^{-6}$ with a sensitivity of 76.9% and specificity of 79.2%. Cats with UNCR values higher than their cutoffs experienced significantly faster deterioration with a median of 19 days.

Conclusions: Both urinary NGAL and UNCR are useful markers for the prediction of CKD progression in cats.

Key words: Azotemia; Biomarker; Feline renal diseases; NGAL; Progression.

Chronic kidney disease (CKD) is gradually progressive and is an irreversible disease in cats. The prevalence of CKD in cats has been reported to range from 1.6 to 20% and the prevalence is increased to 31% in cats >15 years of age.

Evaluation of CKD has mainly relied on testing renal function. Glomerular filtration rate (GFR) is considered to be the best index of renal function. However, determination of GFR requires special equipment and rigorous sampling methods, which are impractical in clinical practice. Serum urea nitrogen and creatinine concentration are commonly used indicators of renal function, but they are insensitive and are influenced by extrarenal factors. Identification of more sensitive, reliable, and practical biomarkers that allow the evaluation of renal diseases in veterinary medicine is warranted.

Neutrophil gelatinase-associated lipocalin (NGAL) is a 25-kDa glycoprotein that was originally isolated from the granules of neutrophils. The protein is expressed during inflammatory responses, but also is increased in urothelial damage when epithelial damage is present.

Abbreviations:

- AUROC: area under ROC
- BUN: blood urea nitrogen
- CKD: chronic kidney disease
- CV: coefficient of variation
- GFR: glomerular filtration rate
- HCT: hematocrit
- HRP: horseradish peroxidase
- IQR: interquartile range
- IRIS: international renal interest society
- MS: mass spectrometry
- NGAL: neutrophil gelatinase-associated lipocalin
- OD: optical density
- PBS: phosphate-buffered saline
- PBST: phosphate-buffered saline with Tween
- pNGAL: plasma neutrophil gelatinase-associated lipocalin
- ROC: receiver operating characteristic curve
- UNCR: urinary NGAL (uNGAL)-to-creatinine ratio
- uNGAL: urinary neutrophil gelatinase-associated lipocalin
- WBC: white blood cells
NGAL in Cats with CKD

Establishing a Sandwich ELISA for the Detection of Feline NGAL

Using a 96-well microtiter plate, anti-dog NGAL mouse antibody at 1:800 dilution was added to each well and incubated at 37°C for 2 hour; this antibody was used as the capture antibody. After incubation, the plate was washed with phosphate-buffered saline (PBS) 3 times and then 150 μL blocking buffer (PBS with Tween [PBST] containing 5% dried milk) was added to each well, which was followed by incubation at 37°C for 1 hour. Test samples (2-fold diluted with PBS), together with 15 serially diluted feline recombinant NGAL calibrators with concentrations ranging from 0 to 27,600 pg/mL, then were individually added and the plate was incubated at 4°C overnight. Each experiment was set up in duplicate. After washing the plate 3 times with PBST, 2,000-fold diluted detector anti-dog NGAL rabbit antibody was loaded and the plate incubated at 37°C for 1 hour. At this point, any unbound antibody was removed and this was followed by washing the plate with PBS. Subsequently, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody diluted 5,000-fold was added to the wells and the plate was incubated for 1 hour. The excess HRP-conjugated goat anti-rabbit IgG antibody then was removed and the plate washed again with PBS. Next, 100 μL tetrathenylbenzidine substrate was added to the wells and the plate was incubated at room temperature in the dark for 10 minutes. Finally, 50 μL of 2M H₂SO₄ was used to terminate the reaction and the optical density (OD) value was measured at a wavelength of 450 nm. The result from the duplicate plates was averaged and the NGAL concentrations were expressed as nanograms per milliliter (ng/mL). Intra- and inter assay assessments were performed to evaluate the precision and repeatability of this in-house ELISA.

Materials and Methods

Preparation of Recombinant Feline NGAL

Initially, the feline NGAL gene (GenBank accession number: XM_003995991.2) was amplified by polymerase chain reaction (PCR); subsequently, it was digested with BamHI/XhoI and cloned into the vector pET32b. Expression and purification of the recombinant feline NGAL protein was carried out by following a procedure described previously. Mass spectrometry (MS) analysis was performed to confirm the authenticity of the feline NGAL recombinant protein. The MS results predicted that the purified protein was neutrophil gelatinase-associated lipocalin-like [Felis catus].

Production and Characterization of Antibodies Against Natural Feline NGAL

The NGAL protein sequences of dogs and cats share 74% identity and therefore it seems likely that antibodies raised using canine NGAL and feline NGAL proteins may cross-react. In our study, 3 kinds of anti-NGAL antibodies, namely anti-dog NGAL rabbit antibodies, anti-dog NGAL mouse antibodies, and anti-cat NGAL mouse antibodies, were generated using procedures described in a previous study. However, the quality and affinity of the anti-cat NGAL antibody against feline NGAL were poorer than that of the rabbit and mouse anti-canine NGAL antibodies when detecting feline NGAL protein as determined by Western blot analysis. As the anti-dog NGAL antibodies did cross-react with feline NGAL, these antibodies were used thereafter for the sandwich ELISAs in our study.

Establishing a Sandwich ELISA for the Detection of Feline NGAL

Concentration also has been shown to be highly associated with progression to end-stage renal diseases. The benefit of using NGAL for the assessment of dogs with renal diseases recently has been reported. The urinary NGAL-to-creatinine ratio (UNCR) also has been found to be an indicator for dogs with acute kidney injury (AKI), CKD, and urinary tract infection (UTI). In addition, serum NGAL (sNGAL) and uNGAL have been identified as useful prognostic markers when evaluating dogs with naturally occurring CKD. Dogs with higher concentrations of sNGAL and uNGAL seem to have shorter survival times.

Although using NGAL to detect CKD is useful in humans, laboratory animals, and dogs, this protein has not yet been tested as a biomarker in cats. We hypothesized that NGAL also is a marker for the evaluation of CKD in cats. The objectives of our study were as follows: (i) to establish an ELISA system for the measurement of NGAL in cats, (ii) to confirm the effectiveness of urine and plasma NGAL in the diagnosis of CKD in cats, (iii) to evaluate the correlation between NGAL in cats and other variables, (iv) to assess the sensitivity and specificity of NGAL in relation to the progression of CKD in cats, and (v) to determine the correlation between NGAL concentrations and the severity of kidney disease in cats.

Patients and Sample Collection

Urine and plasma samples were collected from all cats admitted to the National Taiwan University Veterinary Hospital (Taipei, Taiwan) from September 2014 to March 2016. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of National Taiwan University (Approval No: 103-000084). Urine and serum samples were stored at −80°C until each ELISA was carried out. The diagnosis, clinical information, hematological data, and serum biochemical data of each case also were recorded.

Cats that had persistent azotemia (serum creatinine concentration >1.6 mg/dL for at least 1 month) and with clinical features of CKD (e.g., polyuria, polydipsia, small irregular kidneys, decreased corticomedullary distinction on abdominal ultrasonography) were enrolled. However, cats with urinary obstruction (which were considered to be postrenal azotemia), cats with acute deterioration of azotemia within a month before examination (which was defined as a >0.3 mg/dL increase in serum creatinine concentration within 48 hours), and cats with an infectious disease (such as feline infectious peritonitis) or neoplastic disease were excluded. Based on the IRIS staging system, cats with CKD were classified into 3 groups: (i) IRIS stage 2, (ii) IRIS stage 3, and (iii) IRIS stage 4.

Among the CKD groups, the cases in which the serum creatinine concentration increased by >0.5 mg/dL from baseline within 30 days were defined as undergoing progression. The cats that died or were euthanized because of kidney disease progression also were considered to have undergone progression. Finally, all cases in which it was not possible to follow up for 30 days were excluded from the progression evaluation part of our study.

Healthy cats with no history of kidney and other diseases and among normal results for hematocrit, white blood cell count, segmented neutrophil count, alanine aminotransferase, aspartate transaminase, and alkaline phosphatase as well as serum albumin, total protein, glucose, blood urea nitrogen and creatinine concentrations, as well as normal urinalysis, were enrolled as the control group.
**Statistical Methods**

Statistical software was used for analysis. Initially, the data-sets were analyzed using the Kolmogorov–Smirnov test to determine whether they had a normal distribution. Those datasets with a normal distribution were presented as means ± standard deviation and 1-way analysis of variance (ANOVA) was then used to compare the differences between groups; differences between 2 groups were determined by posthoc testing (the least significant difference test). Those datasets that were not normally distributed were expressed as medians and interquartile range (IQR). The Kruskal–Wallis test and the Mann-Whitney U-test were used to perform nonparametric analysis. In all cases, a P value of <.05 was considered significant. Spearman correlation coefficients were used to evaluate the correlation between NGAL and other variables.

Receiver operating characteristic (ROC) analysis was used to calculate the area under the ROC (AUROC) for pNGAL, sNGAL, and UNCR in the CKD population to determine the most suitable variable for prediction of clinical progression. Sensitivity and specificity were calculated to predict the optimal cutoff of the ROC. Kaplan–Meier curves were applied to assess the 30-day renal progression of the CKD groups. Finally, Cox proportional hazard regression model analysis was applied to determine the hazard ratio associated with the progression of CKD and stepwise forward elimination for the multivariate model to select significant variables.

**Results**

In total, 18 healthy cats and 80 cats with CKD were enrolled. Using the IRIS staging system, the CKD group was divided into 3 subgroups, and these consisted of 26 cats in stage 2, 32 cats in stage 3, and 22 cats in stage 4. Coefficients of variation then were used to evaluate the stability of the ELISA system by repeated analysis of the samples. Intra-assay variation: Calibrators of known concentrations, including low (27 pg/mL), middle (431 pg/mL), and high (3450 pg/mL) concentrations, were measured for 5 times using the same plate on the same day. The coefficient of variation (CV%) for each sample represents the ratio of the standard deviation to the mean. The average of the individual CV was used to represent the intra-assay CV. Interassay variation: The 3 same concentrations of calibrators (i.e., 27, 431, 3450 pg/mL) loaded on different plates were measured on 4 different days to monitor possible plate-to-plate variations. The mean and standard deviation of each concentration for all plates were calculated and then used for calculating the overall CV. The average of the high, middle, and low CV was used to express the precision and repeatability of the assay. The average intra-assay CV was 6% and the average interassay CV was 13%. Therefore, the study procedures showed a good level of performance.

Statistically, the cats with CKD were significantly older and had a lower body weight and a lower hematocrit (HCT) than the control group (P < .05; Table 1). The urinary NGAL concentration and UNCR values of the cats with CKD stage 3 and stage 4 were significantly higher than those of the healthy control cats and the CKD stage 2 cats (P < .001 for both). By way of contrast, there were no significant differences when pNGAL concentration was compared between the CKD groups and the control group. In the control group, UNGAL (Spearman’s correlation, 0.165; P = .609), UNCR (Spearman’s correlation, 0.463; P = .130), and serum creatinine (Spearman’s correlation, −0.172; P = .508) were not correlated with the age of the patients.

In the CKD cats, uNGAL concentration was directly correlated with UNCR values (Spearman’s correlation, 0.917; P < .001). Both uNGAL concentration and UNCR values were directly correlated with serum creatinine concentration, segmented neutrophil count, and serum phosphorous concentration. The exception was UNCR, which showed an inverse significant correlation with HCT (Table 2).

In total, 53 cats with CKD were enrolled with the aim of evaluating progression. Of these, 14 cats experienced a >0.5 mg/dL increase in serum creatinine concentration from baseline within 30 days, and 3 cats died during the observational period. These 17 cats were considered to have undergone progression and formed the progression group; the remaining 36 cats served as the nonprogression group. When these 2 groups were compared, the progression group had higher uNGAL, UNCR, phosphorus, BUN, and creatinine as well as higher urine protein-to-creatinine ratios (UPC) and significantly lower HCT (Table 3).

**Table 1.** The differences in various variables between the control and CKD groups.

| Parameter | Control | CKD2 | CKD3 | CKD4 | P  |
|-----------|---------|------|------|------|----|
| Sex (male) | 66.7% (12/18) | 72% (18/25) | 65.6% (21/32) | 45.5% (10/22) | .270 |
| Age (years) | 4* (7), n = 18 | 8* (9), n = 25 | 14* (4), n = 29 | 14.5* (10), n = 20 | <.001 |
| Body weight (kg) | 4.2* (2.97), n = 17 | 4.1* (1.3), n = 24 | 3.92* (2.0), n = 25 | 3.73* (1.0), n = 18 | .026 |
| HCT (%) | 41.7* (6.35), n = 18 | 33* (9.6), n = 23 | 32* (11.7), n = 31 | 29.95* (7.7), n = 22 | <.001 |
| Serum creatinine | 1.5* (0.35), n = 17 | 2.3* (0.5), n = 26 | 3.6* (1.05), n = 32 | 8.05* (4.15), n = 22 | <.001 |
| concentration (mg/dL) | | | | | |
| uNGAL (ng/mL) | 0.95* (1.35), n = 13 | 0.64* (1.53), n = 19 | 2.49* (2.79), n = 20 | 4.91* (70.3), n = 15 | <.001 |
| pNGAL (ng/mL) | 211.07 (303.29), n = 12 | 171.05 (158.52), n = 22 | 189.83 (210.86), n = 25 | 134.87 (182.26), n = 15 | .303 |
| UNCR (×10−6) | 0.23* (0.59), n = 13 | 0.34* (0.79), n = 19 | 2.97* (10.35), n = 20 | 12.18* (88.81), n = 15 | <.001 |

Data are presented as medians (IQR) and compared by Kruskal–Wallis test and Mann-Whitney U-test. Sex was analyzed using the chi-square test. The different superscripts (a, b, c) indicate various significant differences. HCT, hematocrit; uNGAL, urinary NGAL; pNGAL, plasma NGAL; UNCR, urinary NGAL-to-creatinine ratio.
The AUROC values for UNCR and uNGAL with respect to the prediction of progression were 0.78 and 0.71, respectively. The best cutoff for uNGAL was 2.06 ng/mL, with a sensitivity of 76.9% and a specificity of 75% (P < .05), whereas the best cutoff for UNCR was 4.08 × 10⁻⁶, with a sensitivity of 76.9% and a specificity of 79.2% (P < .05, Fig. 1). The cats with CKD then were divided into a high-value group and a low-value group using the cutoff value of 4.08 × 10⁻⁶ for UNCR on an individual basis. The cats with a UNCR > 4.08 × 10⁻⁶ showed significantly faster progression to endpoint (P < .05). According to the UNCR, the median progression time was 19 days for the high-value group and was 30 days for the low-value group (Fig 2). However, the univariate Cox model analysis indicated that the uNGAL, UNCR, and pNGAL were not significantly associated with an increase in the hazard ratio for progression when other factors, such as HCT, age, and phosphorus, were taken into account. The results of the multivariate Cox model analysis indicate that only phosphorus and HCT were significantly associated (P < .05) with an increase in the hazard ratio for CKD progression (Table 4).

**Discussion**

To our knowledge, our study is the first to investigate the use of uNGAL as a biomarker for cats with naturally occurring kidney disease. The findings show that uNGAL and UNCR may be useful biomarkers for chronic renal damage in cats. The uNGAL concentration and UNCR values of CKD cats were significantly higher than those of healthy cats. Additionally, cats at a higher CKD IRIS stage (stages 3 and 4) had higher uNGAL concentrations and UNCR values than those with CKD IRIS stage 2 and healthy cats. Thus, this increase would seem to be associated with the severity of renal impairment. Additionally, using Spearman’s correlation, UNCR values were found to be significantly associated with a range of factors known to represent the severity of kidney disease, such as serum creatinine and phosphorus concentrations and lower HCT. However, because the uNGAL concentration and UNCR values were not significantly different when CKD IRIS stage 2 and healthy cats were compared, these 2 variables cannot be used to detect early stage of CKD.

The findings from the ROC analysis indicated that uNGAL concentration and UNCR values may be useful for the prediction of clinical progression in cats with CKD. Renal tubular injury has been shown to play an

### Table 2. The correlations between uNGAL, UNCR, and other variables in cats with CKD.

| Correlated Variables | uNGAL | UNCR |
|----------------------|--------|-------|
|                      | Spearman's Correlation | P     | Spearman's Correlation | P     |
| Age                  | 0.246  | .088  | 0.279  | .053               |
| HCT                  | -0.251 | .076  | -0.419 | .002               |
| Segmented neutrophils| 0.310  | .023  | 0.288  | .035               |
| Creatinine           | 0.519  | <.001 | 0.611  | <.001               |
| Phosphorus           | 0.400  | .005  | 0.434  | .002               |
| pNGAL                | 0.006  | .970  | 0.066  | .703               |

HCT, hematocrit; uNGAL, urinary NGAL; pNGAL, plasma NGAL; UNCR, urinary NGAL-to-creatinine ratio.

### Table 3. Differences in variables between cats with CKD that progressed and those that did not progress.

| Parameter          | Nonprogressing Cats | Progressing Cats | P     |
|--------------------|---------------------|------------------|-------|
| Sex (male)         | 55.6% (20/36)       | 64.7% (11/17)    | .7    |
| Age (years)        | 11.5 (5.0), n = 36  | 11.4 (4.8), n = 13 | .202  |
| Weight (kg)        | 4.0 (2.34), n = 27  | 3.88 (0.95), n = 13 | .127  |
| BP (mmHg)          | 136 (56), n = 18    | 156 (45), n = 14  | .985  |
| HCT (%)            | 33.2 (4.7), n = 34  | 28.5 (8.3), n = 16 | .014  |
| WBC (/mm³)         | 8700 (5800), n = 34 | 10600 (15075), n = 16 | .066  |
| Seg (/mm³)         | 5914 (3886.3), n = 34 | 6873.5 (16354.5), n = 16 | .076  |
| Creatinine (mg/dL) | 2.4 (1.3), n = 37   | 5.5 (3.5), n = 16  | <.001  |
| Ca (mg/dL)         | 10.3 (6.6), n = 15  | 8 (5.8), n = 4    | .115  |
| P (mg/dL)          | 4.7 (1.92), n = 34  | 5.6 (4.3), n = 13  | .029  |
| Na (mmol/L)        | 158 (6.8), n = 35   | 157 (6.3), n = 15  | .907  |
| K (mmol/L)         | 4.0 (0.3), n = 35   | 3.93 (0.8), n = 15 | .526  |
| Cl (mmol/L)        | 119 (7.7), n = 35   | 119 (5), n = 15    | .916  |
| uNGAL (ng/mL)      | 0.8 (1.3), n = 24   | 3.1 (67.9), n = 13 | .036  |
| pNGAL (ng/mL)      | 175.5 (181.4), n = 29 | 144 (165), n = 12  | .506  |
| UNCR (<10⁻⁶)       | 0.4 (1.4), n = 24   | 13.9 (119.2), n = 13 | .005  |
| USG                 | 1.01 (0.02), n = 32 | 1.01 (0.003), n = 13 | .003  |
| UPC                 | 0.11 (0.4), n = 12  | 1.05 (1.6), n = 5  | .019  |

*Data are median (IQR) and compared by Student’s t-test.

b Data are mean (SD) and compared by Student’s t-test.

HCT, hematocrit; WBC, white blood cells; Seg, segmented neutrophil count; Ca, calcium; P, phosphorus; uNGAL, urinary NGAL; pNGAL, plasma NGAL; UNCR, urinary NGAL-to-creatinine ratio.

Sex was analyzed using the chi-square test.
important role in the progression of CKD. Neutrophil gelatinase-associated lipocalin is considered to be a tubular biomarker because it is released into urine from injured tubular cells, and uNGAL concentration and UNCR values are useful markers that reflect the severity of a variety of renal diseases. Moreover, the AUROC for the UNCR values (0.78) is higher than that for uNGAL concentration (0.71) in cats with CKD. This finding suggests that UNCR is a better prognostic indicator than uNGAL concentration, which is consistent with findings of a previous report in which it was suggested that urine volume affects uNGAL concentration. Thus, when uNGAL is corrected using urine creatinine concentration to give the UNCR, its utility as a diagnostic tool is enhanced. However, because the increase of UNCR/uNGAL was not significantly associated with the increase of hazard ratio in CKD progression in cats, the capability of UNCR/uNGAL in the
prediction of progression was not as good as HCT and phosphorus.

In our study, pNGAL was not significantly related to CKD in cats. However, in dogs, pNGAL represents a strong and independent marker for predicting the progression of kidney disease. Plasma NGAL originates not only from damaged kidneys but also from other organs. In such circumstances, a concurrent disturbance is consistent with previous studies that have shown high NGAL concentrations. Additional studies are needed to investigate kidney disease. The findings of our study are consistent with a number of previous studies in humans in which it was found that pNGAL was not as sensitive as uNGAL when investigating the progression of renal disease.

The concentrations of pNGAL were found to be much higher than that of uNGAL in both healthy cats and dogs with CKD. This differs from the situation in healthy people, most laboratory animals, and dogs, where only low concentrations of NGAL are detectable in the systemic circulation. This difference suggests that there are certain biological characteristics of cats or of feline NGAL metabolism that are different from those in humans, rodents, and dogs. It is known that NGAL exists in a number of different molecular forms in dogs and humans. In cats, we hypothesize that, unlike other species, pNGAL may come from a variety of cell types and have a number of different molecular forms. Some of these could be expressed at a high constant rate in cats, which would result in a high plasma NGAL concentration. Additional studies are needed to assess this hypothesis. Despite the high concentration of pNGAL in cats, there was poor correlation between pNGAL and uNGAL, which may indicate that the increased concentration of uNGAL in cats may not originate directly from the high expression of circulating NGAL. The issue of high concentration of pNGAL in cats deserves further investigation.

Our results show that the median age of affected cats was 13 years old, whereas the median age of the healthy control group was 4 years old. This significant difference is consistent with previous studies that have shown CKD to be primarily an age-related disease. According to the IRIS staging system, the severity of proteinuria indicates a worse prognosis for the patient and this is consistent with the findings of our study in which an increase in proteinuria was paralleled by the progression of CKD.

In conclusion, uNGAL and UNCR appear to be promising renal biomarkers for kidney disease in cats. Furthermore, CKD cats that have higher concentrations of uNGAL and higher UNCR values tend to have more rapid clinical progression.

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Footnote

* SPSS 20.0 for Windows, SPSS Inc, Chicago, IL

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