Received: 4 December 2019 | Accepted: 26 March 2020

DOI: 10.1111/jvim.15781

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Urine hemojuvelin in cats with naturally occurring kidney disease

Hwei Jing[1,6] | Wei-Li Hsu[2] | Vin-Cent Wu[3] | Han-Ju Tsai[6] | Shang-Feng Tsai[4] | Pei-Shiue Jason Tsai[5] | Tai-Shuan Lai[3] | Ya-Jane Lee[1,6]

[1]Institute of Veterinary Clinical Science, School of Veterinary Medicine, College of Bio-Resources and Agriculture, National Taiwan University, Taipei City, Taiwan

[2]Graduate Institute of Microbiology and Public Health, College of Veterinary Medicine, National Chung-Hsing University, Taichung City, Taiwan

[3]Department of Internal Medicine, National Taiwan University Hospital, Taipei City, Taiwan

[4]Division of Nephrology, Department of Internal Medicine, Taichung Veterans General Hospital, Taichung City, Taiwan

[5]Department of Veterinary Medicine, School of Veterinary Medicine, National Taiwan University, Taipei City, Taiwan

[6]National Taiwan University Veterinary Hospital, College of Bio-Resources and Agriculture, National Taiwan University, Taipei City, Taiwan

Correspondence
Ya-Jane Lee, Institute of Veterinary Clinical Science, School of Veterinary Medicine, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei City, Taiwan.
Email: yajanelee@ntu.edu.tw

Funding information
Ministry of Science and Technology, Taiwan, Grant/Award Number: MOST 107-2313-B-002-053-

Abstract

Background: Soluble-type hemojuvelin in serum and urine has been shown to be a biomarker in humans for chronic kidney disease (CKD) and acute kidney injury (AKI). No similar research has been conducted on cats.

Objective: Urine hemojuvelin (u-hemojuvelin) can be used as a clinical indicator for cats with various renal diseases.

Animals: Eighteen healthy cats, 10 cats with AKI, 21 cats with acute-on-chronic kidney injury (ACKI), and 45 cats with CKD were enrolled.

Methods: The expression profile of u-hemojuvelin was assessed by Western blot analysis, whereas the u-hemojuvelin concentration was measured using an in-house sandwich ELISA. Each cat's u-hemojuvelin-to-creatinine ratio (UHCR) also was determined.

Results: Significant differences were found in both u-hemojuvelin concentration and UHCR between the control cats and the other cats (AKI, CKD, ACKI). Both u-hemojuvelin and UHCR had high areas under the receiver operator curve (AUROC) for diagnoses of AKI (u-hemojuvelin, 0.885; UHCR, 0.982), CKD (hemojuvelin, 0.869; UHCR, 0.959), and ACKI (hemojuvelin, 0.910; UHCR, 1). Late stage (International Renal Interest Society, IRIS stages 3 and 4) CKD cats had significantly higher u-hemojuvelin concentration and UHCR than did early stage cats (IRIS stages 1 and 2). Both u-hemojuvelin and UHCR were significantly correlated with high blood urea nitrogen, plasma creatinine, and plasma phosphate concentrations and with low hematocrit

Abbreviations: Ab, antibody; ACKI, acute on chronic kidney injury; AKI, acute kidney injury; ALP, alkialine phosphatase; ALT, alanine trasmaninase; AST, aspartate transaminase; AUROC, area under ROC; BUN, blood urea nitrogen; CKD, chronic kidney disease; CV, coefficient of variation; GFR, glomerular filtration rate; Hct, hematocrit; IFN, interferon; IQR, interquartile range; NGAL, neutrophil gelatinase-associated lipocalin; OD, optical density; PBST, phosphate-buffered saline with Tween; RBC, red blood cells; ROC, receiver operating characteristics; UHCR, urine hemojuvelin-to-creatinine ratio; u-hemojuvelin, urine hemojuvelin; UPC, urine protein-to-creatinine ratio; USG, urine specific gravity; WBC, white blood cells.

Hwei Jing and Wei-Li Hsu contributed equally to this study.

(Hct), red blood cell (RBC) count, and plasma albumin concentration. The UHCR values were also significantly correlated with white blood cell count in blood.

Conclusion: Both u-hemojuvelin and UHCR potentially can serve as diagnostic indicators for a range of renal diseases in cats.

KEYWORDS

AKI, azotemia, biomarker, cat, CKD, feline

1 | INTRODUCTION

Renal diseases are common and chronic kidney disease (CKD) is usually irreversible in animals.[1] Indirect glomerular filtration rate (GFR) markers, such as serum urea nitrogen and creatinine concentrations, commonly are used as indicators of renal function, but they are relatively insensitive and influenced by extrarenal factors.[2] Therefore, more sensitive, reliable, and practical biomarkers that allow better evaluation of renal disease are warranted.

Hemojuvelin, a glycophoephatidy linositol-linked membrane protein, plays a key regulatory role in systemic iron homeostasis via the hemojuvelin-hepcidin-ferroportin axis.[3] There are 2 isoforms of hemojuvelin: the membrane-bound type and the soluble type. Two cellular proteases are responsible for cleaving membrane-bound hemojuvelin and producing distinguishable soluble isoforms. Cleavage occurs by the action of furin, which produces a 40 kDa protein,[4] and by the action of a type II transmembrane serine protease (TMPRSS6), which yields 2 forms of soluble hemojuvelin, a 15 kDa protein and a 35 kDa protein.[5] However, no animal studies have confirmed these metabolites.

Recently, research has determined that expression of hemojuvelin is significantly increased in the proximal renal tubules when intrinsic renal injury occurs.[6] The concentrations of the soluble types of u-hemojuvelin, when adjusted for using the urine hemojuvelin-to-creatinine ratio (UHCR), have been shown to be early biomarkers for the prediction of acute kidney injury (AKI) and also have been able to predict outcome in human patients after cardiovascular surgery.[7] Urine hemojuvelin (u-hemojuvelin) has been reported to have higher predictive accuracy when detecting early AKI than does neutrophil gelatinase-associated lipocalin (NGAL).[6] Additionally, the serum concentration of hemojuvelin also has been confirmed to be increased in human CKD patients who are undergoing hemodialysis, although it was not increased in nondialysis CKD patients. Furthermore, the increase in serum hemojuvelin concentration seems to be independent of any estimates of patient status based on GFR.[8]

However, no relevant research has been carried out on u-hemojuvelin in cats. We hypothesized that urine soluble-type hemojuvelin also would be able to serve as a diagnostic indicator for various renal diseases in cats. Our objectives were to identify the presence of the soluble forms of hemojuvelin in the urine of cats, to evaluate whether increases in u-hemojuvelin were associated with various kidney diseases of cats, and, finally, to assess whether there is a correlation between u-hemojuvelin and other variables associated with CKD in cats.

2 | MATERIALS AND METHODS

2.1 | Patients and sample collection

Urine samples from cats admitted to the National Taiwan University Veterinary Hospital for clinical evaluation were retrospectively evaluated. The urine samples were stored in a −80°C refrigerator until ELISA analysis. The analysis results, consisting of clinical information, including the cats' history and physical examination results, as well as each animal's hematology, plasma biochemistry, plasma electrolyte concentrations, and urinalysis findings, were recorded. Cats with azotemia (plasma creatinine concentration ≥ 1.6 mg/dL) or with abnormal urinalysis findings (eg, urine specific gravity <1.030) were evaluated first. Any cat with a diagnosis of neoplasia, cardiac disease, increased liver enzyme activity (alkaline phosphatase [ALP], alanine transaminase [ALT], and aspartate transaminase [AST] actvities), infectious disease (eg, pneumonia, pancreatitis, gastroenteritis, feline immunodeficiency virus, feline leukemia virus, and feline infectious peritonitis), neurologic disease, or a lower urinary tract disease, except those with ureteroliths, were excluded. The remaining cases were classified into various groups according to the criteria described below.

The AKI group included cats that showed relevant clinical signs that appeared in the 7 days before presentation, as described by the owner, but without any history of CKD. The relevant clinical history included lethargy, vomiting, and diarrhea. Cats with either oliguria (1-2 mL/kg/h) or anuria (<1 mL/kg/h) after correction of hydration by fluid therapy, also were considered to have AKI.

The CKD group showed at least 1 of the following: a history including relevant clinical signs (eg, polyuria, polydipsia, vomiting, diarrhea), abnormal urinalysis findings (urine specific gravity <1.030, persistent proteinuria based on urine protein-to-creatinine ratio [UPC] > 0.5 on 3 occasions and more than 2 weeks apart or both), small irregular sized kidneys (<2.4 × second lumbar vertebra) or some combination of these. These abnormalities must have lasted at least 30 days. All CKD cases were further grouped according to the International Renal Interest Society (IRIS) staging system into 4 stages. If a cat had a history of CKD and had AKI (an increase in plasma creatinine concentration ≥ 0.5 mg/dL with related clinical signs that had appeared within 7 days), they were included in the acute on chronic kidney injury (ACKI) group.

A control group was recruited and defined as cases without any clinical signs and that also had findings in the reference range for CBC, plasma biochemistry (including AST, ALT, and ALP activities, and plasma albumin, glucose, and total protein concentrations), as well as having

blood urea nitrogen (BUN) and plasma creatinine concentrations within the reference range (BUN concentration < 30 mg/dL and plasma creatinine concentration < 1.6 mg/dL).

2.2 | Preparation of recombinant hemojuvelin protein for antibody (Ab) generation

Sequence alignment indicated the human HFE2 gene (NCBI accession number: NG_011568.1), the canine HFE2 gene (NCBI accession number: XP_022260651), and feline HFE2 gene (NCBI accession number: XP_023114851) showed high sequence homology. Hence, the human HFE2 gene was selected for expression of recombinant protein because of the known stability of this protein. The sequences of primers used for amplification were forward: 5′-TTCATATGCAGGAATGCATTGACCAG and reverse: 5′-TTCTCGAGCTGAATGCAAAGCCACAGAAC. The amplified gene then was cloned into the vector pET24 (Novagen) for expression in *Escherichia coli*. After induction with 0.8 mM of isopropyl thiogalactoside (IPTG), the recombinant hemojuvelin was purified by chromatography using Ni-chelating sepharose (GE Healthcare). The purified recombinant hemojuvelin protein was used to immunize rabbits and rats to generate rabbit-origin and rat-origin antihemojuvelin polyclonal antibodies (Abs); Ab production was carried out by a biotechnology company (Yoa-Hong Biotechnology Inc, Taiwan).

2.3 | Identifying the expression profile of hemojuvelin in feline urine by Western blot analysis

The proteins present in clinical specimens were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (Bio-Rad Laboratories Inc.), followed by electro-transfer to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories Inc).[9] Either rabbit antihemojuvelin serum or rat antihemojuvelin serum, serving as the detection Ab, was reacted individually with the membranes. Ultimately, the signal on the membrane was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitrotetrazolium blue chloride (BCIP/NBT) as substrate (AP Conjugate Substrate Kit, Bio-Rad Laboratories Inc). The final images were captured using an ImageQuant LAS4000 (GE Healthcare, Uppsala, Sweden).

2.4 | Establishing an in-house sandwich ELISA to measure feline u-hemojuvelin concentrations

The ELISA for detecting and measuring u-hemojuvelin was based on a protocol that has been described previously.[9] Briefly, 100 μL/well of the rabbit-origin antihemojuvelin serum, which acted as the capture Ab, was diluted 1:30 in coating buffer (0.035 M $NaHCO_3$ and 0.015 M Na_2CO_3, pH 9.6) and added to the wells of a 96-well ELISA plate (Nunc). The plate then was incubated at 4°C for 16 hours, followed by thorough washing with phosphate-buffered saline with Tween (PBST) buffer (Medicago; 100 μL per well) 3 times. Each plate then was blocked with PBST buffer containing 5% skimmed milk (100 μL per well) at 37°C for 1 hour. Next, diluted urine samples, as well as serially diluted calibrator samples (recombinant hemojuvelin protein), were individually added to each well (100 μL per well) and the plate incubated at 4°C for 16 hours. All samples were analyzed in duplicate. After washing the plate with PBST buffer 3 times, each well was loaded with 100 μL of the 3000-fold diluted detector Ab (rat-origin antihemojuvelin serum). Next, the plate was incubated at 37°C for 2 hours after which the above washing procedure was carried out again. After this washing, diluted horseradish peroxidase conjugated goat-origin ant-rat secondary Ab was added to each well (100 μL per well) and the plate incubated for 1 hour at 37°C. Signal was developed after addition of tetramethylbenzidine (TMB; 100 μL per well; BD Biosciences) for 30 minutes at room temperature in the dark. At this point, the reaction was stopped by adding 2 N H_2SO_4 (50 μL per well). The optical density (OD) 450_{nm} of each well was measured immediately using a microplate ELISA reader (TECAN). The concentration of hemojuvelin was expressed as picograms per milliliter (pg/mL). Three individual calibrator samples containing known concentrations of hemojuvelin with low, middle and high OD values were measured 4 times on the same day on the same plate and these results were used to assess intra-assay precision. At the same time, the OD values of the same 3 calibrators were measured on 4 different plates to assess interassay precision. The coefficient of variation (CV, %) for each calibrator was calculated as the ratio of the SD of the means. The average values of the respective CVs represented the intra-assay CV and the interassay CV.

2.5 | Statistical analyses

The statistical software Statistical Product and Service Solutions (SPSS 20 for Windows) was used in this study. Initially, the Shapiro-Wilk test was used to determine normality. Normally distributed data sets are presented as mean with SD, and then 1-way analysis of

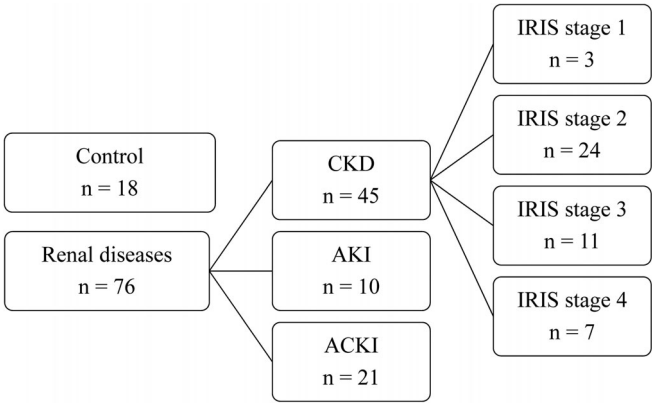

FIGURE 1 The case groupings used in our study. The renal disease cats were the cases with azotemia (creatinine ≥1.6 mg/dL) or with an abnormal urinalysis (eg, urine specific gravity < 1.030) and excluded any cases with a diagnosis of neoplasm, cardiac disease, an increase in ALP, ALT, and AST, an infectious disease, a neurological disease, or a lower urinary tract diseases. The control cases were recruited with healthy cats

TABLE 1 Clinical characteristics and biochemical variables of the various groups

Variables	Control		CKD		AKI		ACKI		*P*
u-Hemojuvelin (pg/mL)	33.37[a] (31.11, 36.67)	n = 18	41.71[b] (37.6, 54.9)	n = 45	39.74[b] (38.07, 76.81)	n = 10	50.08[b] (39.43, 62.95)	n = 21	<.001*
u-UHCR (10^{-7})	0.096[a] (0.065, 0.127)	n = 17	0.664[b] (0.256, 1.102)	n = 45	0.764[b] (0.385, 1.246)	n = 10	0.895[b] (0.543, 1.571)	n = 21	<.001*
Age (years)	3.5[a] (1.0, 9.0)	n = 18	10.0[b] (6.5, 14.0)	n = 45	9.0[ab] (5.8, 14.3)	n = 10	12.5[b] (9.3, 16.0)	n = 20	.001*
Sex (male)	41.2%	(7/17)	53.3%	24/45	50.0%	5/10	52.4%	11/21	.857
Body weight (kg)	4.29 (3.85, 5.01)	n = 12	3.74 (3.10, 4.80)	n = 43	3.16 (2.14, 5.61)	n = 9	3.75 (2.90, 5.10)	n = 20	.362
Hct (%)	43.60[a] (42.20, 46.10)	n = 15	33.45[b] (26.63, 39.50)	n = 40	29.90[bc] (23.78, 37.43)	n = 8	25.70[c] (20.55, 32.15)	n = 21	<.001*
RBC counts ($\times 10^6/\mu L$)	9.91[a] (8.70, 10.29)	n = 11	7.23[b] (6.04, 8.57)	n = 40	6.70[bc] (5.48, 8.29)	n = 8	6.11[c] (4.93, 7.24)	n = 19	<.001*
WBC counts (/μL)	8000[a] (6500, 10 200)	n = 15	7750[a] (5300, 10 675)	n = 40	18 650[b] (12 600, 27 925)	n = 8	11 200[ab] (7850, 12 700)	n = 21	.001*
Albumin (g/dL)	3.50[a] (3.20, 3.60)	n = 13	3.20[ab] (2.90, 3.50)	n = 31	3.45[ab] (3.05, 3.58)	n = 8	3.00[b] (2.70, 3.30)	n = 21	.014*
Total protein (g/dL)	7.50 (7.15, 7.90)	n = 13	7.50 (7.18, 8.1)	n = 26	7.55 (6.65, 8.35)	n = 6	7.75 (7.15, 8.45)	n = 16	.842
BUN (mg/dL)	20.5[a] (19.0, 21.3)	n = 18	36.0[b] (25.0, 57.0)	n = 45	87.0[bc] (20.5, 159.5)	n = 10	141.0[c] (124.5, 185.5)	n = 19	<.001*
Creatinine (mg/dL)	1.55[a] (1.30, 1.70)	n = 18	2.30[b] (1.90, 4.30)	n = 45	4.60[bc] (2.25, 13.53)	n = 10	10.50[c] (7.05, 15.95)	n = 21	<.001*
Phosphate (mg/dL)	4.75[a] (4.30, 4.88)	n = 8	4.75[a] (4.28, 6.00)	n = 42	4.90[ab] (3.50, 14.00)	n = 9	11.10[b] (7.60, 13.50)	n = 19	<.001*
Sodium (mmol/L)	155.40 (150.85, 157.60)	n = 5	153.80 (152.10, 156.40)	n = 43	153.20 (151.15, 155.70)	n = 10	150.50 (144.65, 154.95)	n = 20	.101
Potassium (mmol/L)	3.44 (3.27, 3.79)	n = 5	3.83 (3.61, 4.18)	n = 43	3.84 (3.40, 4.77)	n = 10	4.04 (2.74, 4.73)	n = 20	.440
Chloride (mmol/L)	114.90 (114.75, 120.80)	n = 5	117.80 (116.10, 119.10)	n = 43	118.30 (112.33, 121.95a)	n = 10	113.35 (109.10, 119.18)	n = 20	.210
USG	1.057[a] (1.035, 1.062)	n = 18	1.012[b] (1.009, 1.019)	n = 45	1.012[b] (1.010, 1.020)	n = 10	1.010[b] (1.008, 1.013)	n = 21	<.001*
Urine pH	6.48[a] (6.01, 6.61)	n = 18	5.79[b] (5.55, 6.39)	n = 45	5.73[ab] (5.47, 5.94)	n = 10	5.55[b] (5.33, 5.85)	n = 21	<.001*

Note: The variables were analyzed by the Kruskal-Wallis test and the Dunn-Bonferroni test. The values are shown as medians and IQR in brackets. Sex is analyzed by χ^2 square test. The different superscripts (a, b, c) indicate the various levels of significant difference among groups (adjusted $P < .05$).

Abbreviations: ACKI, acute-on-chronic kidney injury; AKI, acute kidney injury; BUN, blood urea nitrogen; CKD, chronic kidney disease; Hct, hematocrit; RBC, red blood cells; UHCR, urine hemojuvelin-to-creatinine ratio; u-hemojuvelin, urine hemojuvelin; USG, urine specific gravity; WBC, white blood cells.

*$P < .05$.

variance (ANOVA) with post hoc Bonferroni test or Student's *t* test was applied to compare the difference among groups or between groups. The Mann-Whitney *U* test or Kruskal-Wallis test with post hoc Dunn test and Bonferroni error correction were used for nonparametric analysis, and the nonparametric data are presented as medians with interquartile ranges (IQRs). Categorical data are presented as proportions; they were tested using the χ^2 test or Fisher's exact test when comparing the data sets. Receiver operating characteristic (ROC) curves were used to assess the ability of variables to differentiate the different renal diseases or different IRIS stages of CKD. Furthermore, the best cutoff values, which were defined as the maximum sum of sensitivity and specificity, also were determined to identify values that had the best specificities and sensitivities. Spearman correlation was used to analyze the correlation between different variables. A *P* value < .05 was used to identify results that were significantly different.

3 | RESULTS

Ninety-four cats were enrolled in the study. There were 18 cats in control group. Forty-four cats were assigned to the CKD group, which was further subclassified into stage 1 (3 cats), stage 2 (24 cats), stage 3 (11 cats), and stage 4 (7 cats) groups. The AKI group consisted of 10 cats, whereas the ACKI group consisted of 21 cats (Figure 1).

Two subtypes of soluble hemojuvelin were detected in the feline urine samples. One subtype had a molecular weight between 25 and 35 kDa and was designated the large subtype, and the other had a molecular weight between 15 and 25 kDa and was designated the small subtype. Furthermore, 3 concentrations of soluble hemojuvelin were used as calibrators, namely 438.5, 109.7, and 13.7 pg/mL) to evaluate the precision of the in-house sandwich ELISA established in the study. The average intra-assay CV was 4.7%, and the average interassay CV was 3.4%. These findings indicate that this procedure performs well and that the internal controls were adequate.

Statistically, cats in both the CKD and ACKI groups were significantly older than the control group (*P* < .05; Table 1), but no significant difference was found in the sex distributions among the various groups. The AKI group had significantly higher white blood cells count than the other groups. The case group, including CKD, AKI, and ACKI, had significantly higher medians for BUN and plasma phosphate concentration, as well as lower medians for hematocrit (Hct), red blood cells (RBC) and urine specific gravity, compared to the control group. The CKD and ACKI groups also had significantly lower urine pH than the control group.

When we compared all of the renal disease groups together with the control group, the former had significantly higher concentration of u-hemojuvelin and UHCR (median [IQR], 33.37 [31.11, 36.67] pg/mL and 0.096 [0.065, 0.127]) $\times 10^{-7}$, respectively; Table 1). However, no significant differences were found in u-hemojuvelin and UHCR values among the CKD (41.71 [37.6, 54.9] pg/mL and 0.664 [0.256, 1.102] $\times 10^{-7}$), AKI (39.74 [38.07, 76.81] pg/mL and 0.765 [0.385, 1.246] $\times 10^{-7}$) and ACKI (50.08 [39.43, 62.95] pg/mL, and 0.895 [0.543, 1.571] $\times 10^{-7}$) groups.

Both u-hemojuvelin and UHCR showed no significant differences when these values were compared individually across the 4 IRIS stages of CKD (data not shown). However, when stages 1 and 2 were combined and defined as early stage CKD, and stages 3 and 4 were combined as late stage CKD, significant differences for both u-hemojuvelin and UHCR were found between these 2 combination groups (u-hemojuvelin, 38.4 [35.7, 44.9] pg/mL of the early stage vs 55.3 [42.6, 106.3] pg/mL of the

TABLE 2 Clinical characteristics and biochemical variables of the control group and of the early and late stage CKD groups

Variables	Control		Early stage		Late stage		*P*
u-Hemojuvelin (pg/mL)	33.37[a] (31.11, 36.67)	n = 18	38.44[b] (35.65, 44.94)	n = 27	55.33[c] (42.6, 106.30)	n = 18	<.001*
u-UHCR (10^{-7})	0.096[a] (0.065, 0.127)	n = 17	0.355[b] (0.207, 0.668)	n = 27	1.102[c] (0.698, 2.703)	n = 18	<.001*
Age (year-old)	3.5[a] (1.0, 9.0)	n = 18	8.0[a] (4.0, 13.0)	n = 27	13.5[b] (10.8, 15.0)	n = 18	<.001*
Hct (%)	43.60[a] (42.20, 46.10)	n = 15	37.00[b] (26.90, 41.10)	n = 23	28.60[c] (22.25, 34.10)	n = 17	<.001*
RBC counts ($\times 10^6$/μL)	9.91[a] (8.70, 10.29)	n = 11	8.43[b] (6.30, 9.43)	n = 23	6.36[c] (4.54, 7.35)	n = 17	<.001*
Albumin (g/dL)	3.50[a] (3.20, 3.60)	n = 13	3.20[ab] (3.05, 3.50)	n = 17	3.20[b] (2.70, 3.43)	n = 14	.049
BUN (mg/dL)	20.5[a] (19.0, 21.3)	n = 18	28.0[a] (21.0, 36.0)	n = 27	64.0[b] (43.8, 115.3)	n = 18	<.001*
Creatinine (mg/dL)	1.55[a] (1.30, 1.70)	n = 18	2.10[b] (1.80, 2.30)	n = 27	4.80[c] (3.45, 8.55)	n = 18	<.001*
Phosphate (mg/dL)	4.75[ab] (4.30, 4.88)	n = 8	4.30[a] (3.95, 4.95)	n = 25	6.00[b] (4.75, 11.20)	n = 17	.001*
Potassium (mmol/L)	3.44[a] (3.27, 3.79)	n = 5	3.83[a] (3.62, 4.16)	n = 25	3.77[a] (3.49, 4.41)	n = 18	.167
USG	1.057[a] (1.035, 1.062)	n = 18	1.017[b] (1.010, 1.024)	n = 27	1.010[b] (1.007, 1.013)	n = 18	<.001*
Urine pH value	6.48[a] (6.01, 6.61)	n = 18	5.94[a] (5.64, 6.79)	n = 27	5.58[b] (5.43, 5.87)	n = 18	.001*
Urine protein-creatinine ratio	0.06[a] (0.00, 0.19)	n = 4	0.12[ab] (0.09, 0.30)	n = 13	0.82[b] (0.65, 1.18)	n = 7	.028*

Note: The variables were analyzed by Kruskal-Wallis test and Dunn test and are shown as medians with the IQR in brackets. Sex was analyzed by χ^2 square test. The different superscripts (a, b, c) indicated various levels of significant difference. Early stage CKD includes IRIS CKD stages 1 and 2, while late stage CKD includes IRIS CKD stages 3 and 4.

Abbreviations: BUN, blood urea nitrogen; CKD, chronic kidney disease; Hct, hematocrit; IRIS, International Renal Interest Society; RBC, red blood cells; UHCR, urine hemojuvelin-to-creatinine ratio; u-hemojuvelin, urine hemojuvelin; USG, urine specific gravity.

*P < .05.

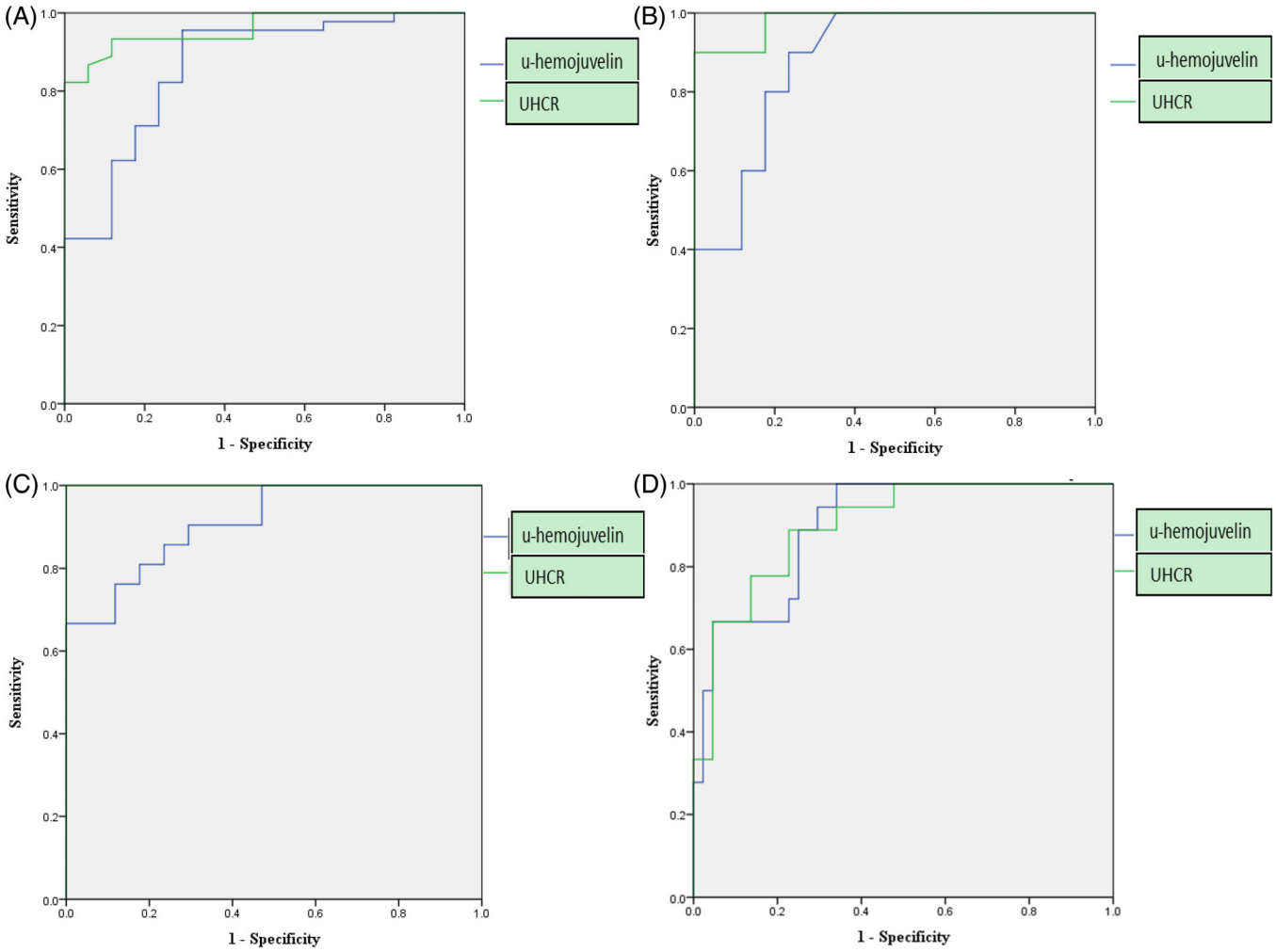

FIGURE 2 Receiver operator characteristic (ROC) curves for u-hemojuvelin and UHCR when the diagnosis is CKD. A, AUROC of u-hemojuvelin: 0.863, 95% CI: 0.758-0.967, the best cutoff of 34.84; AUROC of UHCR: 0959, 95% CI: 0.915-1.000, the best cutoff of 0.224, AKI. B, AUROC of u-hemojuvelin: 0.885, 95% CI: 0.813-1.000, the best cutoff of 36.4; AUROC of UHCR: 0885, 95% CI: 0.954-1.000, the best cutoff of 0.286, and ACKI. C, AUROC of u-hemojuvelin: 0.91, 95% CI: 0.823-0.998, the best cutoff of 44.82; AUROC of UHCR: 1.0 95% CI: 1-1, the best cutoff of 0.272; when differentiating between early and late stage CKD. D, AUROC of u-hemojuvelin: 0.895, % CI: 0.821-0.975, the best cutoff of 38.96; AUROC of UHCR: 0.899, 95% CI: 0.820-0.978, the best cutoff of 0.440. ACKI, acute-on-chronic kidney injury; AKI, acute kidney injury; AUROC, area under ROC; CKD, chronic kidney disease; UHCR, urine hemojuvelin-to-creatinine ratio; u-hemojuvelin, urine hemojuvelin

TABLE 3 Correlations between u-hemojuvelin, UHCR, and various variables for all enrolled cases

u-Hemojuvelin	Spearman correlation	*P*	UHCR	Spearman correlation	*P*
Body weight	−0.126	.253	Body weight	−0.259	.017*
Hct	−0.375	<.001*	Hct	−0.564	<.001*
RBC count	−0.337	.003*	RBC count	−0.512	<.001*
WBC count	−0.009	.935	WBC count	0.174	.114
Neutrophils	0.174	.115	Neutrophils	0.354	0.001*
Albumin	−0.271	.020*	Albumin	−0.291	.013*
BUN	0.390	<.001*	BUN	0.566	<.001*
Creatinine	0.494	<.001*	Creatinine	0.685	<.001*
Phosphate	0.286	.011*	Phosphate	0.480	<.001*
Sodium	0.015	.895	Sodium	−0.087	.451
Potassium	0.184	.107	Potassium	0.256	.024*
Chloride	0.160	.162	Chloride	−0.016	.886

(Continues)

TABLE 3 (Continued)

u-Hemojuvelin	Spearman correlation	*P*	UHCR	Spearman correlation	*P*
USG	−0.478	<.001*	USG	−0.848	<.001*
Urine pH	−0.328	.001*	Urine pH	−0.392	<.001*

Abbreviations: ACKI, acute-on-chronic kidney injury; AKI, acute kidney injury; BUN, blood urea nitrogen; CKD, chronic kidney disease; Hct, hematocrit; RBC, red blood cells; UHCR, urine hemojuvelin-to-creatinine ratio; u-hemojuvelin, urine hemojuvelin; USG, urine speficic gravity; WBC, white blood cells.
*$P < .05$.

late stage; UHCR, 0.4 [0.2, 0.7] pg/mL of the early stage vs 1.1 [0.7, 2.7] $\times 10^{-7}$ of the late stage, respectively; Table 2).

The ROC curve analysis of both u-hemojuvelin and UHCR for the diagnosis of CKD, AKI and ACKI gave significant results ($P \leq .001$) and there was a high areas under the receiver operator curve (AUROC) when differentiating the control group from the CKD group (AUROC, 0.868 for u-hemojuvelin and 0.959 for UHCR) from the AKI group (AUROC, 0.885 for u-hemojuvelin and 0.982 for UHCR) and from the ACKI group (AUROC, 0.910 for u-hemojuvelin and 1 for UHCR). This was also true when differentiating early stage CKD from late stage CKD (AUROC, 0.898 for u-hemojuvelin and 0.899 for UHCR; Figure 2). The best cutoff values also were predicted.

Both u-hemojuvelin and UHCR were significantly and negatively correlated with Hct, RBC count, plasma albumin concentration, urine specific gravity, and urine pH, whereas they were positively correlated with plasma BUN, plasma creatinine, and plasma phosphate concentration, and UPC. The UHCR also was significantly negatively correlated with body weight, neutrophil count, and plasma potassium concentration. However, the positive correlation between UHCR and plasma potassium concentration existed only in the acute azotemia group (AKI and ACKI; Spearman correlation, 0.374; $P = .042$; Table 3).

4 | DISCUSSION

To our knowledge, ours is the first study to identify urinary hemojuvelin in cats and also to investigate the role in cats of urinary hemojuvelin in the presence of naturally occurring renal disease.

Cats with AKI or ACKI had significantly higher concentrations of u-hemojuvelin and higher UHCR compared to control cats, which is similar to humans with AKI.[7] The common causes of intrinsic AKI in cats include nephrotoxins and ischemia[10] and these also are common causes of AKI in humans.[11] An increase in u-hemojuvelin has been reported to result from abnormal iron homeostasis,[4] and it has been shown that in humans that hemojuvelin is upregulated markedly in the proximal renal tubules in response to injuries.[7] These include cast formation, tubular dilatation and tubular necrosis,[6,7] and consequently can result in iron deposition and further damage to the renal tubules.[6] Considering that the previous results for humans and our present results for cats are very similar, it seems likely that the increase in u-hemojuvelin found in cats with AKI originates from injured renal tubules. Because tubulointerstitial lesions are common in cats with CKD,[1] the increase of u-hemojuvelin and UHCR in CKD cats also may originate from the renal tubules.

Although in our study, using the Spearman test, u-hemojuvelin, and UHCR in cats were found to be significantly correlated with plasma creatinine concentration, which is an indirect GFR indicator,[12] they did not show a linear relationship. Specifically, no significant result was found when linear regression between u-hemojuvelin or UHCR and plasma creatinine concentration was carried out (see supplementary information), even after log transformation analysis (data not shown). Furthermore, it would seem that u-hemojuvelin and UHCR cannot be used together to differentiate AKI from CKD, and therefore more research is needed on these variables as potential markers of renal injury.

In our study, u-hemojuvelin and UHCR were significantly and negatively correlated with a range of common indicators of anemia, including Hct and RBC count. In CKD, the etiology of anemia includes inflammation, which causes shortened RBC survival, and impaired iron metabolism because of deficiencies in hepcidin and erythropoietin.[13] In other studies, hemojuvelin in blood has been shown to be related to iron metabolism by its influence on the hepcidin-ferroportin axis[3,14] and modulation of various cytokines.[15,16] Some evidence suggests that soluble hemojuvelin is increased by iron deficiency and is decreased during iron overload.[17] Thus, it is possible that these effects might be associated with the demand for iron in anemic CKD cats. However, additional variables related to iron metabolism during CKD, such as serum ferritin, transferrin saturation, and total iron binding capacity,[18] need to be investigated simultaneously in future studies to understand the cross-talk among the above variables in cats.

If we examine the role of hemojuvelin in immunity, the protein's presence has been shown to influence the level of interleukin-6 expression,[16] and thus hemojuvelin likely would be able to modulate the production of various cytokines, including interferon (IFN)-γ by macrophages.[16] A previous study found that interleukin-6 and IFN-γ together have an influence on leukocyte trafficking during inflammation.[19] Such involvement might help to explain the finding that UHCR has a significant positive correlation with neutrophil count.

Urine pH also was found to be negatively correlated with u-hemojuvelin. Renal dysfunction often leads to metabolic acidosis,[20] which likely is the cause of the acidic urine.[21] Thus, this negative correlation might be an effect of impaired renal function. Nevertheless, the mechanism behind the acidification of the urine still is worthy of further study.

The UHCR was found to have a significant positive correlation with plasma potassium concentration. This correlation was found in the AKI and ACKI groups. Hyperkalemia is a common clinical finding in AKI and ACKI in humans[11] and cats,[22] and it is a result of decreased renal excretion.[11] This correlation implies that UHCR may be an

indicator of an acute episode of renal impairment, which is similar to the findings in humans.[6,7]

Our study shows that u-hemojuvelin and UHCR were significantly increased when various renal diseases were present in cats. Nevertheless, they have not been compared to other renal biomarkers for CKD in cats. These include serum symmetric dimethylarginine, which has been reported be a more sensitive GFR marker than serum creatinine concentration,[23] NGAL, which has been shown to be a tubular injury biomarker,[9] and fibroblast growth factor 23, which has been shown to be related to hyperphosphatemia.[24] A comparison of these biomarkers with u-hemojuvelin and UHCR is warranted in the future to understand their various roles in the range of different renal diseases that affect cats.

Our study had some limitations. First, the low number of AKI cases and the timing of urine collection might have weakened the significance of the findings in this group. In studies of humans, the timing of sample collection after renal or cardiac surgery was within 24 hours,[6,7] and significant increases in hemojuvelin could be detected as early as 3 hours after surgery.[7] Thus, it is possible that we missed the peak of u-hemojuvelin in our cats with AKI, which would have resulted in decreased sensitivity. Second, some of the control cats in our study had slightly higher plasma creatinine concentrations than typically used for IRIS CKD staging. However, they were all healthy and <2 years-old, as well as having normal results for their urinary examinations. Their muscle mass was good, which might be the reason behind the increase in creatinine concentration in both plasma and urine.[21] Nonetheless, without a direct measure of GFR, renal function cannot be directly assessed to confirm that the control cats were healthy.

In our study, many factors may have affected the results because they cannot be excluded. First, because the number of control cases was not large enough to establish a normal reference range for u-hemojuvelin and UHCR, the influence of age on our findings cannot be evaluated. Additionally, because the expression and release of soluble hemojuvelin seems to occur in different organs independently[25] and seems to occur during inflammation,[4] diseases other than renal disease, such as hyperthyroidism, neoplasia, and cardiovascular disease, also may have influenced u-hemojuvelin and UHCR. In addition, hydration status could not be assessed for the cats in our study, and thus prerenal factors cannot be excluded. Finally, biopsies to evaluate pathological diagnoses were not performed in our study, and thus the specific causes of renal disease in the individual cats could not be determined.

5 | CONCLUSIONS

Urine hemojuvelin and UHCR are potential renal biomarkers for cats when assessing different kidney diseases. These markers also can be correlated with the severity of CKD.

ACKNOWLEDGMENTS

The authors thank all the doctors at National Taiwan University Veterinary Hospital for providing the cases and collecting the samples. Our study was fully supported by Ministry of Science and Technology, Taiwan (MOST 107-2313-B-002-053). Part of our study was presented at 2nd Asian Small Animal Specialist Veterinary Congress 2019.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Our study received IACUC approval (NTU106-EL-00208; approval date April 2018).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for our study.

ORCID

Ya-Jane Lee https://orcid.org/0000-0003-4884-7019

REFERENCES

1. Reynolds BS, Lefebvre HP, Feline CKD. Pathophysiology and risk factors – what do we know? *J Feline Med Surg*. 2013;15(Suppl. 1):3-14.
2. Finch N. Measurement of glomerular filtration rate in cats: methods and advantages over routine markers of renal function. *J Feline Med Surg*. 2014;16:736-748.
3. Zhang AS. Control of systemic iron homeostasis by the hemojuvelin-hepcidin axis. *Adv Nutr*. 2010;1:38-45.
4. Silvestri L, Pagani A, Camaschella C. Furin-mediated release of soluble hemojuvelin: a new link between hypoxia and iron homeostasis. *Blood*. 2008;111:924-931.
5. Silvestri L, Pagani A, Nai A, de Domenico I, Kaplan J, Camaschella C. The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. *Cell Metab*. 2008;8:502-511.
6. Young GH, Huang TM, Wu CH, et al. Hemojuvelin modulates iron stress during acute kidney injury: improved by furin inhibitor. *Antioxid Redox Signal*. 2014;20:1181-1194.
7. Ko SW, Chi NH, Wu CH, et al. Hemojuvelin predicts acute kidney injury and poor outcomes following cardiac surgery. *Sci Rep*. 2018;8:1938.
8. Rumjon A, Sarafidis P, Brincat S, et al. Serum hemojuvelin and hepcidin levels in chronic kidney disease. *Am J Nephrol*. 2012;35:295-304.
9. Wang IC, Hsu WL, Wu PH, Yin HY, Tsai HJ, Lee YJ. Neutrophil gelatinase-associated lipocalin in cats with naturally occurring chronic kidney disease. *J Vet Intern Med*. 2017;31:102-108.
10. Worwag S, Langston CE. Acute intrinsic renal failure in cats: 32 cases (1997-2004). *J Am Vet Med Assoc*. 2008;232:728-732.
11. Rahman M, Shad F, Smith MC. Acute kidney injury: a guide to diagnosis and management. *Am Fam Physician*. 2012;86:631-639.
12. Rule AD, Larson TS, Bergstralh EJ, Slezak JM, Jacobsen SJ, Cosio FG. Using serum creatinine to estimate glomerular filtration rate: accuracy in good health and in chronic kidney disease. *Ann Intern Med*. 2004;141:929-937.
13. Chalhoub S, Langston C, Eatroff A. Anemia of renal disease: what it is, what to do and what's new. *J Feline Med Surg*. 2011;13:629-640.
14. Kautz L, Nemeth E. Novel tools for the evaluation of iron metabolism. *Haematologica*. 2010;95:1989-1991.
15. Fleming RE. Iron and inflammation: cross-talk between pathways regulating hepcidin. *J Mol Med (Berl)*. 2008;86:491-494.
16. Wu Q, Shen Y, Tao Y, et al. Hemojuvelin regulates the innate immune response to peritoneal bacterial infection in mice. *Cell Discov*. 2017;3:17028.

17. Core AB, Canali S, Babitt JL. Hemojuvelin and bone morphogenetic protein (BMP) signaling in iron homeostasis. *Front Pharmacol*. 2014;5:104.
18. Hayes W. Measurement of iron status in chronic kidney disease. *Pediatr Nephrol*. 2019;34:605-613.
19. McLoughlin RM, Witowski J, Robson RL, et al. Interplay between IFN-gamma and IL-6 signaling governs neutrophil trafficking and apoptosis during acute inflammation. *J Clin Invest*. 2003;112:598-607.
20. Sparkes AH, Caney S, Chalhoub S, et al. ISFM consensus guidelines on the diagnosis and management of feline chronic kidney disease. *J Feline Med Surg*. 2016;18:219-239.
21. Baxmann AC, Ahmed MS, Marques NC, et al. Influence of muscle mass and physical activity on serum and urinary creatinine and serum cystatin C. *Clin J Am Soc Nephrol*. 2008;3:348-354.
22. Ross L. Acute kidney injury in dogs and cats. *Vet Clin North Am Small Anim Pract*. 2011;41:1-14.
23. Relford R, Robertson J, Clements C. Symmetric dimethylarginine: improving the diagnosis and staging of chronic kidney disease in small animals. *Vet Clin North Am Small Anim Pract*. 2016;46:941-960.
24. Geddes RF, Finch NC, Elliott J, Syme HM. Fibroblast growth factor 23 in feline chronic kidney disease. *J Vet Intern Med*. 2013;27:234-241.
25. Rodriguez Martinez A, Niemela O, Parkkila S. Hepatic and extrahepatic expression of the new iron regulatory protein hemojuvelin. *Haematologica*. 2004;89:1441-1445.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Jing H, Hsu W-L, Wu V-C, et al. Urine hemojuvelin in cats with naturally occurring kidney disease. *J Vet Intern Med*. 2020;34:1222–1230. https://doi.org/10.1111/jvim.15781