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Assessment of cell cycle arrest biomarkers and neutrophil gelatinase-associated lipocalin to distinguish acute kidney injury from other diseases in dogs

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Abstract

Background: Cell cycle arrest biomarkers (tissue inhibitor of metalloproteinase-2 [uTIMP-2] and insulin-like growth factor binding protein 7 [uIGFBP7]), and neutrophil gelatinase-associated lipocalin (NGAL) variables are valuable biomarkers for early diagnosis of acute kidney injury (AKI) in people.

Objectives: To evaluate uTIMP-2, uIGFBP7, fractional excretion of NGAL (FeNGAL), and urinary to serum NGAL ratio (u/sNGAL) in healthy dogs, dogs with AKI, dogs with chronic kidney disease (CKD), and critically ill (CI) dogs.

Animals: Forty-two client-owned dogs (healthy, n = 10; AKI, n = 11; CKD, n = 11; CI, n = 10).

Methods: Prospective, observational study. After assessment of routine renal biomarkers, stress (uTIMP-2, uIGFBP7) and damage (NGAL) biomarkers were measured, using ELISA kits, and normalized to urinary creatinine (uCr).

Results: Normalized uTIMP-2 and [uTIMP-2] × [uIGFBP7]/uCr were significantly higher in the AKI group (median 151.9 [range, 2.2-534.2] and 62.9 [1.1-266.8] pg/mL respectively), compared to healthy dogs (0.3 [0.2-74.7]; $P < .001$ and 0.16 [0.1-58.1] pg/mL; $P < .001$), dogs with CKD (0.7 [0.3-742.5]; $P = .04$ and 0.37 [0.2-180.1] pg/mL; $P = .03$) and CI dogs (1.9 [0.2-37.0]; $P = .03$ and 0.8 [0.1-16.1] pg/mL; $P = .02$). Fractional excretion of NGAL was significantly higher in dogs with AKI (54.17 [7.93-155.32] %), than in healthy (0.03 [0.01-0.21] %; $P < .001$) and CI dogs (3.05 [0.05-28.86] %; $P = .02$).

Abbreviations: [uTIMP-2] × [uIGFBP7], product of urinary tissue inhibitor of metalloproteinase-2 and insulin-like growth factor binding protein 7; AKI, acute kidney injury; APPLE, acute patient physiologic and laboratory evaluation; CI, critically ill; CKD, chronic kidney disease; FeNGAL, fractional excretion of neutrophil gelatinase-associated lipocalin; GFR, glomerular filtration rate; IRIS, International Renal Interest Society; NGAL, neutrophil gelatinase-associated lipocalin; sCr, serum creatinine; SDMA, symmetric dimethylarginine; sNGAL, serum neutrophil gelatinase-associated lipocalin; sUrea, serum ureum; u/sNGAL, urinary to serum neutrophil gelatinase-associated lipocalin; uCr, urinary creatinine; uIGFBP7, urinary insulin-like growth factor binding protein 7; uNGAL, urinary neutrophil gelatinase-associated lipocalin; u_{norm}/sNGAL, urinary to serum neutrophil gelatinase-associated lipocalin with normalized NGAL; u_{norm}NGAL, normalized urinary neutrophil gelatinase-associated lipocalin; UPC, urinary protein : creatinine ratio; USG, urine specific gravity; uTIMP-2, urinary tissue inhibitor of metalloproteinase-2.

Conclusions and Clinical Importance: Normalized uTIMP-2, [uTIMP-2] × [uIGFBP7]/uCr, and FeNGAL can be valuable renal biomarkers for early diagnosis of AKI in dogs.

KEYWORDS
canine, early diagnosis, insulin-like growth factor binding protein 7 (IGFBP7), renal disease, symmetric dimethylarginine (SDMA), tissue inhibitor of metalloproteinase-2 (TIMP-2)

1 | INTRODUCTION

Current diagnosis of acute kidney injury (AKI) in dogs is based on clinical signs, laboratory abnormalities (azotemia, isosthenuric urine), and compatible ultrasonographic findings. The routinely used laboratory changes result from a decrease in glomerular filtration rate (GFR), making them unvaluable for detecting AKI when the injury is not (yet) associated with a measurable decrease in GFR. Detection of non-azotemic AKI or *International Renal Interest Society* (IRIS) grade 1 AKI remains a major diagnostic challenge, although early recognition could allow timely intervention and improve outcome.[1-5]

The urinary cell cycle arrest biomarkers, tissue inhibitor of metalloproteinase-2 (uTIMP-2), and insulin-like growth factor binding protein 7 (uIGFBP7), are stress biomarkers that are superior to all other biomarkers for prediction and early detection of AKI in people when used in a 2-panel combination[6,7] but have not yet been investigated in dogs.

Neutrophil gelatinase-associated lipocalin (NGAL) is a low molecular weight protein, which is highly expressed during ischemic renal injury. It is the most researched "damage" renal biomarker in human and veterinary medicine.[8-16] An important limitation is its concurrent release from circulating neutrophils and consequently its increase in critically ill (CI) patients, irrespective of possible renal disease.[17-19] In people, the urinary to serum NGAL ratio (u/sNGAL) and fractional excretion of NGAL (FeNGAL) are able to differentiate between patients with AKI and chronic kidney disease (CKD).[20]

Serum symmetric dimethylarginine (SDMA) is a marker of declining GFR or so-called "functional renal biomarker," that has mainly been investigated in companion animals with CKD[21-23] and is commonly used in veterinary practice.[21-23] To our knowledge, its diagnostic potential in hospital-acquired AKI is not thoroughly investigated.

The main objective of this prospective study was to evaluate uTIMP-2 and uIGFBP7 in healthy dogs, dogs with CKD, dogs with AKI, and CI dogs. Furthermore, the potential of u/sNGAL and FeNGAL in differentiating dogs with AKI from CKD and CI dogs was assessed. Finally, SDMA was compared between the same dog groups.

2 | MATERIALS AND METHODS

This prospective observational study was conducted at the Small Animal Clinic, Ghent University and was approved by the local Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (Belgium) and by the deontological committee of the Belgian Federal Agency for the Safety of the Food Chain (EC 2020/067; approval date November 19, 2020).

2.1 | Study cohort

Healthy dogs, dogs with CKD, AKI, and CI dogs were enrolled after obtaining signed informed owner consent. Healthy dogs were actively recruited. The AKI, CKD, and CI dogs were included at admission if they met the inclusion criteria, and if it was practically feasible to perform the sampling. In all dogs, general physical examination, noninvasive blood pressure measurement, complete blood count, serum biochemistry profile, urinalysis (including bacterial urine culture), and abdominal ultrasonography were performed to assess their eligibility for this study. In dogs with CKD, AKI, and CI dogs, additional diagnostics were performed at the discretion of the responsible clinician. Exclusion criteria for all dogs were a bodyweight of <2 kg, severe thrombocytopenia (<20.000 plt/µL), coagulation disorders, and severe anemia (hematocrit <13%).

Dogs were considered to be healthy based on their history and absence of clinically relevant abnormalities on physical examination, blood examination, urinalysis, and medical imaging (abdominal ultrasonography and thoracic radiographs). All dogs had to be free from medications (except for preventive medication) for at least 2 months before inclusion.

Diagnosis of CKD was based on clinical history (pu/pd, vomiting, hyporexia, weight loss) and abnormal serum creatinine (sCr; >1.8 mg/dL [>159 µmol/L]; upper reference limit in-house IDEXX Catalyst Dx chemistry analyzer [IDEXX Laboratories Inc, Westbrook, Maine]) in combination with isosthenuric urine. Evidence of chronicity was provided by medical records, compatible ultrasonographic findings (such as irregular renal shape, small kidneys, unclear corticomedullary differentiation, and cortical hyperechogenicity),[24] or a combination of both. Administration of drugs potentially influencing renal perfusion (such as angiotensin converting enzyme inhibitors, angiotensin-receptor blockers, dopamine, calcium channel blockers), were not allowed at least 10 days before inclusion. Dogs with CKD were excluded if they had concurrent systemic disease potentially influencing the kidneys.

Dogs were diagnosed with AKI if they presented with an acute onset of clinical signs (<14 days) and met at least 2 of the following criteria: acute onset of renal azotemia with sCr >1.8 mg/dL (>159 µmol/L; upper reference limit in-house IDEXX Catalyst Dx chemistry analyzer [IDEXX Laboratories Inc, Westbrook, Maine]) persisting minimally 24 hours after correction of prerenal factors, signs of acute tubular injury on urinalysis (glucosuria in the absence of

hyperglycemia or urinary casts), imaging findings compatible with AKI (such as normal renal size or renomegaly, perirenal effusion, perirenal steatitis), progressive non-azotemic increase in sCr (>0.3 mg/dL [>26.5 μmol/L]) within 48 hours and persistent oliguria or anuria over 6 hours after correction of dehydration. Exclusion criteria were a history or ultrasonographical findings compatible with CKD or treatment with drugs influencing renal perfusion (see above) within 10 days before inclusion. All AKI dogs underwent testing for leptospiral infection by performing a serum microagglutination test and real-time quantitative PCR on urine.

To be included in the CI group, dogs had to be admitted to the emergency department because of a potentially life-threatening disease and have an acute patient physiologic and laboratory evaluation score (APPLE fast score) of ≥18. Dogs with clinicopathological or ultrasonographical signs of AKI (as defined above), CKD or post-renal azotemia were excluded.

2.2 | Sample collection

Blood and urine samples were collected as soon as possible and within 4 hours of admission. Blood (10 mL) was obtained by standard venipuncture in the vena jugularis, using a 21G needle and was subsequently divided in serum, heparin and EDTA tubes. Concurrent urine samples (10 mL) were collected by cystocentesis (method of choice), catheterization or spontaneous voiding. Samples were preferably taken at the same time point and before intravenous fluid therapy, but in certain cases (eg, anuric dogs, clinically unstable dogs), the urine sampling was delayed for a maximum of 2 hours after initial stabilization and thus during fluid therapy. In-house analyses were performed within 60 minutes from collection. Urine and serum samples were subsequently sent for external laboratory analyses (urinalysis and routine assessment of kidney function, including SDMA) within 48 hours. The remaining urine supernatant and serum were stored in aliquots of 300 μL at −80°C within 24 hours for a maximum of 1 year before biomarker analyses.

2.3 | Routine assessment of kidney function, including SDMA measurement

Serum was analyzed (IDEXX Bioanalytics, Kornwestheim, Germany) to assess sCr, urea (sUrea), SDMA, albumin, total protein, potassium, calcium, inorganic phosphate, chloride, and sodium. Serum creatinine was measured using Jaffe's kinetic method without deproteinization (manufacturer Beckman Coulter). Quantification of SDMA concentration was performed using the validated immunoassay IDEXX SDMA Test (IDEXX Laboratories Inc, Westbrook, Maine). Values >14 μg/dL were considered abnormal. Urinalysis was performed by Sonic Healthcare Benelux (Belgium) and consisted of urine specific gravity (USG), dipstick analysis, urinary protein : creatinine ratio (UPC), and bacterial urine culture. Additionally, microscopic sediment analysis was performed on-site within 1 hour after collection. For UPC determination, urinary protein concentration was measured by turbidimetry and urinary creatinine (uCr) by spectrophotometry (using Jaffe's kinetic method) with Cobas c702 (Roche Diagnostics). Results of these external laboratories were reported and used for statistical analysis (not the results of in-house laboratory machines).

2.4 | Immunoassays of urinary TIMP-2 and urinary IGFBP7

Before measurement, urine aliquots were thawed for 2 hours at room temperature. Concentrations of uTIMP-2 and uIGFBP7 were measured in single, using the canine TIMP-2 ELISA (Abcam, Cambridge, UK) and canine IGFBP7 ELISA (MyBioSource Inc, San Diego, USA) kits. Validation of these assays was performed by the companies for canine serum. For the present study, the intra-assay coefficient of variation (CV), sensitivity and matrix interference (spiking, linearity) were assessed in canine urine (see Supplemental Information and Table S1). Values below the detection limit were noted as half of the lower limit of detection, multiplied by the dilution factor. The product of uTIMP-2 and uIGFBP7 ([uTIMP-2] × [uIGFBP7]) was calculated by multiplying both absolute values (pg^2/mL^2).[25] The absolute values and the product of the cell cycle arrest biomarkers were normalized to uCr before statistical analyses.[26] Both ELISA assays were performed according to the manufacturer's procedure and are shortly described below.

To measure uTIMP-2, standards (between 20 and 5000 pg/mL) and 1 : 2 diluted urine samples were pipetted into a 96-well plate coated with a specific antibody for canine uTIMP-2, after which the wells were washed and biotinylated anti-canine TIMP-2 antibody was added. After a second wash to remove the unbound biotinylated antibody, Streptavidin-Horseradish Peroxidase was pipetted into the wells. The wells were washed again before adding a 3,3',5,5'-tetramethylbenzidine substrate solution, which caused color to develop in proportion to the amount of bound canine TIMP-2. After 30 minutes, stop solution was added and the intensity of the yellow color was measured at 450 nm.

For IGFBP7, standards (between 6.25 and 200 ng/mL) and undiluted urine samples were pipetted into a 96-well plate coated with a specific antibody for canine IGFBP7. After adding HRP-conjugate reagent, the plate was incubated for 60 minutes (at 37°C). All wells were washed 4 times and chromogen solutions were added. After 15 minutes, stop solution was added and the intensity of the yellow color was measured within 15 minutes at 450 nm.

2.5 | Urinary and serum NGAL analyses

Aliquots of urine and serum were thawed for 2 hours at room temperature before the dilution process and NGAL analyses. Both urinary NGAL (uNGAL) and serum NGAL (sNGAL) were measured in single using a commercially available, canine NGAL ELISA kit (Dog NGAL ELISA Kit, Bioporto, Hellerup, Denmark), following the manufacturer's instructions which are briefly described below. This assay detects values ranging from 0.56 to 400 pg/mL.

Samples were diluted as followed: 1 : 100 for serum and 1 : 10 for urine of healthy dogs; higher dilutions up to 1 : 500 for serum and 1 : 10 000 for urine were used for CI, CKD, and AKI dogs based on their UPC.[16] After dilution, 100 µL of each sample was added to a 96-microwell plate, coated with mouse monoclonal antibodies against canine NGAL. Bound NGAL was detected using a biotin-labeled mouse monoclonal secondary antibody. The immunoassay was developed with HRP-streptavidin and a chromogenic tetramethyl-benzidine substrate. After adding the stop solution, absorbance was measured at a wavelength of 450 nm (by Multiskan FC Microplate Photometer). Concentrations were calculated using a 4-parameter logistic curve fitting program (Skanit software, Thermo Scientific, Germany). The results of uNGAL and sNGAL are presented as absolute concentrations and uNGAL was subsequently normalized to uCr and expressed as u_{norm}NGAL.

Additional variables were calculated:

1. Urinary to serum NGAL ratio with uNGAL (u/sNGAL) = uNGAL/sNGAL.
2. Urinary to serum NGAL ratio with u_{norm}NGAL (u_{norm}/sNGAL) = (uNGAL/uCr)/sNGAL.
3. Fractional excretion of NGAL (FeNGAL) = (uNGAL/sNGAL)/(uCr/sCr) × 100.

2.6 | Statistical analyses

Statistical analyses were performed using a statistical software package (SPSS Statistics 28, IBM, Armonk, USA). Normality of variables was assessed using Shapiro-Wilk tests. Differences in variables between groups were assessed with one-way ANOVA tests for parametric data, whereas Kruskal-Wallis tests were used for nonparametric data. When statistical differences between groups were present ($P \leq .05$), multiple comparison tests with Bonferroni correction (statistically significant if $P \leq .01$) were performed. Following tests with Bonferroni correction, adjusted *P*-values (multiplied by 4 and therefore statistically significant if $P \leq .05$) are reported. Correlations between different variables were evaluated using Spearman correlation tests. Results were considered statistically significant if *P* was ≤.05. Correlations were described as negligible, weak, moderate, strong, or very strong if correlation coefficients were respectively between 0.00-0.10, 0.10-0.39, 0.40-0.69, 0.70-0.89, and 0.90-1.00.[27] A valid power analysis was not possible before the study for the cell cycle arrest biomarkers as no data were available in dogs. Based on data in literature (expected SDMA, sNGAL, and u_{norm}NGAL concentrations were respectively 8.5 µg/dL, 10 ng/mL, and 0.5 ng/mg in healthy dogs; 40 µg/dL, 45 ng/mL, and 180 ng/mg in AKI dogs; 35 µg/dL, 20 ng/mL, and 5 ng/mg in CKD dogs[16,28-31]) and a power of 0.8 and α 0.05, the pre-study sample size was determined to be minimum 6 dogs in each group to detect group differences using one-way ANOVA tests. To avoid nonsignificant results because of differences in study groups between the present and previous studies, we aimed to include at least 10 dogs in each group. Outliers were included in the statistics because of small group size.

3 | RESULTS

3.1 | Study cohort and routine assessment of kidney function

The study cohort comprised 10 healthy dogs, 11 dogs with CKD, 11 dogs with AKI, and 10 CI dogs belonging to a variety of breeds. Relevant clinicopathological data of the 4 groups are listed in Table 1. Age and body weight did not differ significantly between groups. One healthy dog had glucosuria at moment of sampling, which was not detected in both previous and follow-up urinalyses. Furthermore, 1 healthy dog had a mildly elevated sUrea with a normal sCr (0.93 mg/dL) and well-concentrated urine (USG 1.050).

The CKD group comprised 9 dogs diagnosed with IRIS stage 2 (3 hypertensive dogs with proteinuria, 2 normotensive dogs with proteinuria, 1 hypertensive dog without proteinuria, and 3 normotensive dogs without proteinuria) and 2 dogs diagnosed with IRIS Stage 3 (1 dog with hypertension and proteinuria and 1 normotensive dog without proteinuria).

According to the IRIS grading system for AKI, 2 dogs were diagnosed with IRIS grade 2, 3 dogs with IRIS grade 3, 2 dogs with IRIS grade 4, and 3 dogs with IRIS grade 5. Diagnoses in the AKI group included leptospirosis (n = 5), hypercalcemia (n = 1), and pyelonephritis (n = 1). An additional 2 dogs were strongly suspected of leptospirosis but were not confirmed by PCR or serology. In 2 dogs, the cause of AKI remained unknown.

The CI group consisted of 5 dogs diagnosed with acute hemorrhagic diarrhea syndrome, and 1 dog each with acute hepatopathy, septic abdomen, immune-mediated hemolytic anemia, Amanita intoxication with severe hepatopathy, and diabetes ketoacidosis. One CI dog had mild azotemia (2.3 mg/dL) at presentation with a USG of 1.039. Because sCr normalized within 24 hours (0.9 mg/dL), the azotemia was classified as prerenal azotemia, and this dog was not excluded.

Routine renal biomarkers (sCr and sUrea) were higher in the AKI group compared to healthy ($P < .001$ for both) or CI ($P < .001$ and $P = .007$) dogs but were not significantly different between dogs with AKI and CKD ($P = .76$ and $P = 1.0$). The UPC was higher in dogs with AKI ($P < .001$) and CKD ($P = .04$) compared to healthy dogs. Noninvasive systolic blood pressure was higher in dogs with CKD ($P = .005$) than in CI dogs.

3.2 | Cell cycle arrest renal biomarkers uTIMP-2 and uIGFBP7

Concentrations of absolute and normalized uTIMP-2 and uIGFBP7 and their product [uTIMP-2] × [uIGFBP7] for the different groups are shown in Table 2 and Figure 1A-F. Results for uTIMP-2 measurements were available for 41 dogs because of insufficient volume of urine in 1 dog with CKD, whereas results for uIGFBP7 were available for all included dogs (n = 42).

Normalized uTIMP-2 values were higher in the AKI group, when compared to healthy dogs ($P < .001$), dogs with CKD ($P = .03$) and CI

TABLE 1 Clinical and clinicopathological data for all study groups.

Variable (unit) [reference interval]	Healthy n = 10	CKD n = 11	AKI n = 11	CI n = 10
Age (years)	4.6 ± 1.9	8.6 ± 5.1	8.0 ± 2.9	6.5 ± 3.8
Body weight (kg)	20.6 (3.7-38.8)	14.2 (3.9-44.6)	25.9 (8.7-41.0)	8.0 (3.2-30.6)
Noninvasive systolic blood pressure (mm Hg)	140.3 ± 12.0	162.3 ± 38.4[a]	138.1 ± 42.9	112.5 ± 18.9[b]
sCr (mg/dL) [.5-1.8]	.9 (.6-1.6)[c]	2.3 (1.5-4.2)[d]	4.4 (1.8-17.6)[a,d]	.8 (.5-2.3)[b,c]
sUrea (mmol/L) [3.2-10.3]	4.8 (2.11-11.8)[b,c]	15.3 (7.5-37.4)[d]	28.1 (12.1-98.3)[a,d]	5.9 (1.9-33.8)[c]
SDMA (µg/dL) [0-14]	12 (7-14)[b,c]	25 (17-63)[d]	33 (14->100)[a,d]	13 (9-46)[c]
Potassium (mmol/L) [3.9-5.8]	4.2 ± .2	4.9 ± .6	4.6 ± .6	4.2 ± .8
Sodium (mmol/L) [142-153]	147 (145-149)	148 (146-163)	144 (133-156)	147 (132-157)
Chloride (mmol/L) [106-120]	113 (109-117)	113 (107-119)	105 (86-116)	113 (83-119)
Phosphorus (mmol/L) [.9-1.7]	1.1 (.7-1.4)[a,c]	1.5 (.9-4.4)[c]	3.9 (1.5-8.0)[b,d]	1.6 (1.1-2.3)[c,d]
Calcium (mmol/L) [2.1-2.9]	2.5 ± .1[a,b]	2.8 ± .3[a,c]	2.5 ± .3[b]	2.2 ± .1[b,d]
Albumin (g/L) [28-43]	31.7 ± 4.0[a,c]	29.0 ± 3.2[a]	24.4 ± 5.3[d]	23.2 ± 4.9[b,d]
Total protein (g/L) [54-76]	64.3 ± 7.6[a]	62.0 ± 5.5[a]	55.2 ± 14.3	48.6 ± 10.0[b,d]
USG [1.015-1.045]	1.037 ± .01[b,c]	1.016 ± .004[a,d]	1.015 ± .005[a,d]	1.031 ± .01[b,c]
UPC [<.50]	.07 (.05-.11)[b,c]	.37 (.07-5.67)[d]	.84 (.17-10.15)[d]	.28 (.23-8.00)
uCr (mg/dL)	266.0 ± 65.5[a,b,c]	85.4 ± 40.3[d]	59.0 ± 33.1[d]	142.6 ± 124.7[d]
Glucosuria in absence of hyperglycemia	1/10	0/11	6/11	2/10

Note: Results are expressed as mean ± SD for parametric data and median (range) for nonparametric data.
Abbreviations: AKI, acute kidney injury; CI, critically ill; CKD, chronic kidney disease; sCr, serum creatinine; SDMA, symmetric dimethylarginine; sUrea, serum urea; uCr, urinary creatinine; UPC, urinary protein : creatinine ratio; USG, urine specific gravity.
[a]Adjusted $P \leq .05$ compared to CI.
[b]Adjusted $P \leq .05$ compared to CKD.
[c]Adjusted $P \leq .05$ compared to AKI.
[d]Adjusted $P \leq .05$ compared to healthy group.

TABLE 2 Results of cell cycle arrest biomarkers analysis with absolute and normalized values for uTIMP-2, uIGFBP7 and their product.

Variables (unit)	Healthy n = 10	CKD n = 11	AKI n = 11	CI n = 10
uTIMP-2 (pg/mL)	5.1 (≤5.1-2630.0)	5.1 (≤5.1-5940.0)[a,b]	772.0 (≤5.1-4758.0)[c,d]	5.1 (≤5.1-296.2)[a]
uTIMP-2/uCr × 10^{-8}	.3 (.2-74.7)[a]	.7 (.3-742.5)[a,b]	151.9 (2.2-534.2)[c,d,e]	1.9 (.2-37.0)[a]
uIGFBP7 (pg/mL) × 10^{3}	46.7 (28.9-77.7)	49.8 (24.3-90.4)	48.9 (41.4-79.4)	42.1 (37.2-51.3)
uIGFBP7/uCr × 10^{-3}	.2 (.1-.3)[a,c]	.5 (.3-1.7)[e]	1.1 (.5-2.4)[e]	.6 (.1-1.8)
[uTIMP-2] × [uIGFBP7] (pg/mL)^2 × 10^{3}	330.1 (146.3-204 313)	1013 (827-144 045)[b]	34 294.5 (248-287 859)[d]	215.5 (188.4-14 106)[a]
[uTIMP-2] × [uIGFBP7]/uCr (pg/mL) × 10^{-3}	.16 (.1-58.1)[a]	.37 (.2-180.1)[a,b]	62.9 (1.1-266.8)[c,d,e]	.8 (.1-16.1)[a]

Note: Results are expressed as median (range).
Abbreviations: [uTIMP-2] × [uIGFBP7], product of uTIMP-2 and uIGFBP7; AKI, acute kidney injury; CI, critically ill; CKD, chronic kidney disease; uCr, urinary creatinine; uIGFBP7, urinary insulin-like growth factor binding protein 7; uTIMP-2, urinary tissue inhibitor of metalloproteinase-2.
[a]Adjusted $P \leq .05$ compared to AKI.
[b]Missing value.
[c]Adjusted $P \leq .05$ compared to CKD.
[d]Adjusted $P \leq .05$ compared to CI.
[e]Adjusted $P \leq .05$ compared to healthy group.

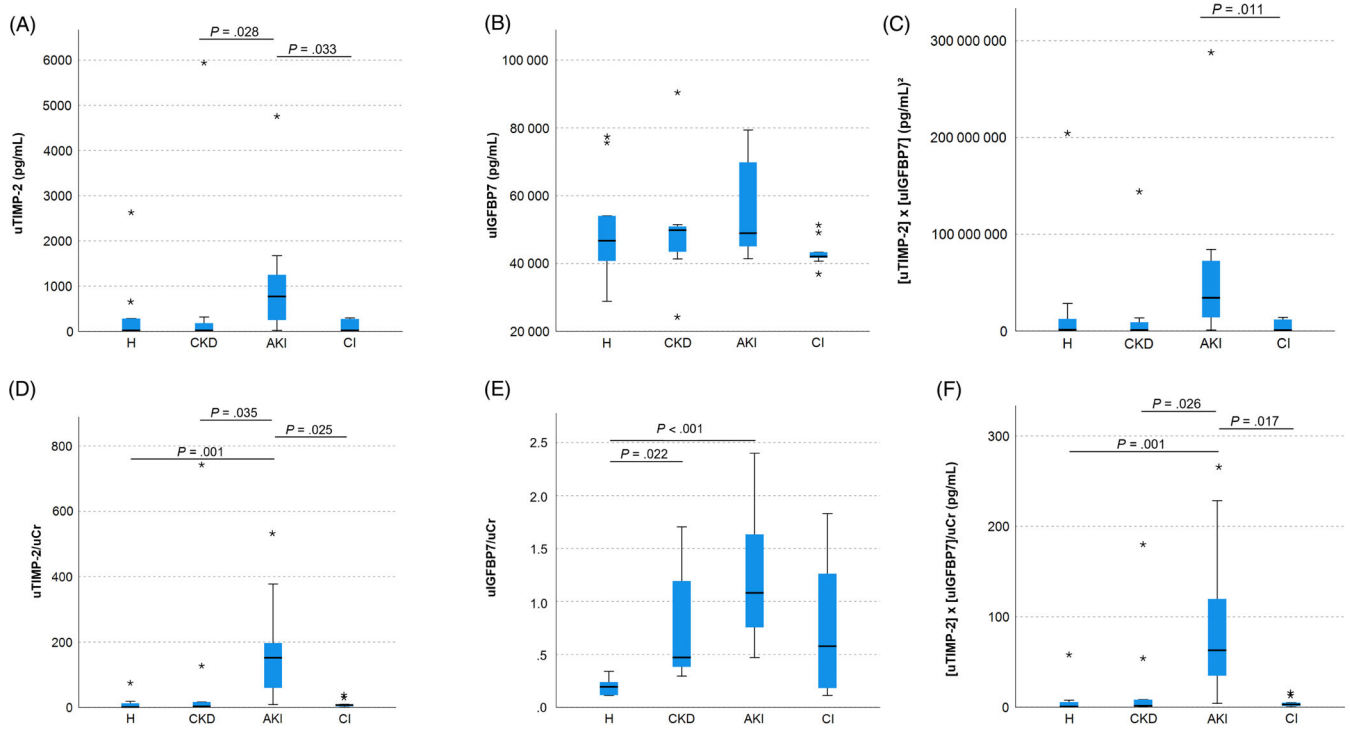

FIGURE 1 Results of cell cycle arrest biomarkers analyses with absolute (A-C) and normalized (D-F) values for uTIMP-2, uIGFBP7, and [uTIMP-2] × [uIGFBP7] respectively. Bolt horizontal line: median; blue box: area between quartile 1 and 3; whiskers: upper and lower bound 1.5-3 times outside the interquartile range; asterisk: outliers (>3 times quartile 1 or 3); adjusted *P*-values are reported. [uTIMP-2] × [uIGFBP7], product of uTIMP-2 and uIGFBP7; AKI, acute kidney injury; CI, critically ill; CKD, chronic kidney disease; H, healthy; uCr, urinary creatinine; uIGFBP7, urinary insulin-like growth factor binding protein 7; uTIMP-2, urinary tissue inhibitor of metalloproteinase-2.

TABLE 3 Correlation coefficients between cell cycle arrest biomarkers and the current commercially available renal biomarkers.

	uTIMP-2 n = 41	uTIMP-2/uCr n = 41	uIGFBP7 n = 42	uIGFBP7/uCr n = 42	[uTIMP-2] × [uIGFBP7] n = 41	[uTIMP-2] × [uIGFBP7]/uCr n = 41
sCr	.421[a]	.499[b]	.370[c]	.423[a]	.465[a]	.509[b]
sUrea	.371[c]	.497[b]	ns	.522[b]	.437[a]	.508[b]
SDMA	ns	.343[c]	ns	.421[a]	ns	.354[c]
USG	ns	−.386[c]	ns	−.771[b]	ns	−.383[a]
UPC	ns	.387[c]	ns	.488[b]	ns	.395[a]

Note: ns refers to not significant ($P > .05$).
Abbreviations: [uTIMP-2] × [uIGFBP7], product of uTIMP-2 and uIGFBP7; sCr, serum creatinine; SDMA, symmetric dimethylarginine; sUrea, serum urea; uCr, urinary creatinine; uIGFBP7, urinary insulin-like growth factor binding protein 7; UPC, urinary protein : creatinine ratio; USG, urine specific gravity; uTIMP-2, urinary tissue inhibitor of metalloproteinase-2.
[a].001 < $P \leq .01$.
[b]$P \leq .001$.
[c].01 < $P \leq .05$.

dogs ($P = .02$). However, 2 CKD dogs (both IRIS stage 2) and 2 healthy dogs also had high normalized TIMP-2 values, whereas their medical records did not reveal evidence of AKI. The uIGFBP7 and uNGAL values of these patients were not distinctively higher than those of the other dogs in their respective patient groups. Normalized uIGFBP7 was significantly lower in healthy dogs compared to azotemic dogs (AKI [$P < .001$] and CKD [$P = .02$]). The normalized [uTIMP-2] × [uIGFBP7] was significantly higher in dogs with AKI compared to healthy dogs ($P < .001$), dogs with CKD ($P = .04$) and CI dogs ($P = .01$).

The cell cycle arrest biomarkers showed mild to moderate positive correlations with sCr, sUrea, and UPC and mild positive correlations with SDMA. Moderate to strong negative correlations were observed between the normalized cell cycle arrest biomarker values and USG. The correlation coefficients are listed in Table 3.

3.3 | Urine and serum NGAL

The results of NGAL biomarker analyses are summarized in Table 4. Results for uNGAL are not available for 1 healthy dog and 1 dog with CKD because of insufficient urine sample volume. Additionally, sNGAL results are missing for 1 dog with AKI and 1 dog with CKD because of insufficient serum sample volume.

Urinary NGAL and u_{norm}NGAL were significantly lower in healthy dogs compared to other groups (respectively AKI $P < .001$, CKD $P = .01$, CI $P = .01$ and AKI $P < .001$, CKD $P = .02$, CI $P = .04$). Serum NGAL was higher in dogs with AKI than in healthy ($P < .001$) and CI dogs ($P = .02$). The u/sNGAL and u_{norm}/sNGAL were significantly lower in the group of healthy dogs compared to the diseased groups. Fractional excretion of NGAL was higher in dogs with AKI than in healthy ($P < .001$) and CI ($P = .02$) dogs. Figure 2A-C illustrates group differences for u/sNGAL, u_{norm}/sNGAL, and FeNGAL respectively.

Moderate positive correlations were found for uNGAL, u_{norm}NGAL, sNGAL, u/sNGAL, and u_{norm}/sNGAL with sCr, sUrea, SDMA,

TABLE 4 Results of uNGAL, u_{norm}NGAL, sNGAL, u/sNGAL, u_{norm}/sNGAL, and FeNGAL in the 4 study groups.

Variables (unit)	Healthy n = 9	CKD n = 10	AKI n = 11	CI n = 10
uNGAL (ng/mL)	1.10 (.12-5.18)[a,b,c]	62.60 (9.59-212.60)[d]	177.90 (30.2-1979)[d]	48.70 (1.98-1180.40)[d]
u_{norm}NGAL[e]	.36 (.04-3.60)[a,b,c]	100.35 (6.19-260.09)[d]	502.47 (67.20-4602.33)[d]	64.15 (.88-1735.88)[d]
sNGAL (ng/mL)	8.40 (3.97-31.92)[a,f]	31.35 (14.67-65.66)	61.06 (14.92-269.50)[a,c,f]	15.07 (3.29-75.38)[a]
u/sNGAL	.93 (<.01-.86)[a,b,c]	2.55 (.34-4.34)[d]	5.08 (.98-12.67)[d,f]	2.92 (.14-29.74)[d]
u_{norm}/sNGAL[g] (mL/ng)	.37 (.01-2.32)[a,b,c]	30.94 (2.17-122.84)[d]	79.72 (28.12-248.52)[d,f]	42.83 (.64-296.63)[d]
FeNGAL (%)	.03 (<.01-.21)[a,b]	5.84 (.49-21.40)[d]	54.17 (7.93-155.32)[c,d,f]	3.05 (.05-28.86)[a]

Note: Values are presented as median (range).
Abbreviations: AKI, acute kidney injury; CI, critically ill; CKD, chronic kidney disease; FeNGAL, fractional excretion of NGAL; sNGAL, serum NGAL; u/sNGAL, urinary to serum NGAL ratio; uNGAL, urinary neutrophil gelatinase-associated lipocalin (NGAL); u_{norm}/sNGAL, u/sNGAL with u_{norm}NGAL; u_{norm}NGAL, uNGAL normalized to urinary creatinine.
[a]Adjusted $P \leq .05$ compared to AKI.
[b]Adjusted $P \leq .05$ compared to CKD.
[c]Adjusted $P \leq .05$ compared to CI.
[d]Adjusted $P \leq .05$ compared to healthy group.
[e]$\times 10^{-6}$.
[f]n = 10.
[g]$\times 10^{-10}$.

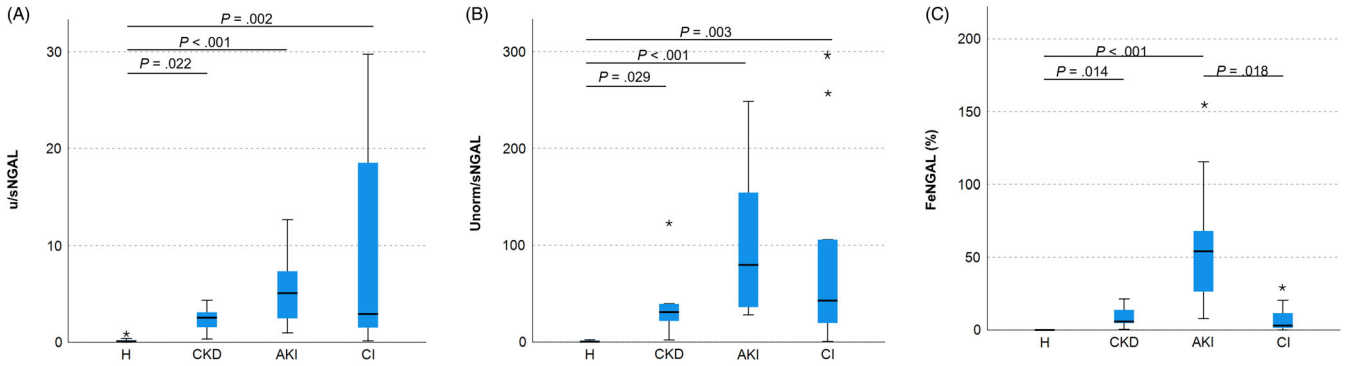

FIGURE 2 Results for u/sNGAL (A), u_{norm}/sNGAL (B), and FeNGAL (C) in all patient groups. Bolt horizontal line: median; blue box: area between quartile 1 and 3; whiskers: upper and lower bound 1.5-3 times outside the interquartile range; asterisk: outliers (>3 times quartile 1 or 3); adjusted *P*-values are reported. AKI, acute kidney injury; CI, critically ill; CKD, chronic kidney disease; FeNGAL, fractional excretion of NGAL; H, healthy; NGAL, neutrophil gelatinase-associated lipocalin; u/sNGAL, urinary to serum NGAL ratio; u_{norm}/sNGAL, u/sNGAL with uNGAL normalized to urinary creatinine.

	uNGAL n = 40	u_{norm}NGAL n = 40	sNGAL n = 40	u/sNGAL n = 39	u_{norm}/sNGAL n = 39	FeNGAL n = 39
sCr	.542[a]	.569[a]	.626[a]	.325[b]	.368[b]	.695[a]
sUrea	.663[a]	.704[a]	.534[a]	.563[a]	.632[a]	.845[a]
USG	ns	−.463[c]	−.445[c]	ns	−.440[c]	−.561[a]
UPC	.690[a]	.689[a]	.483[c]	.629[a]	.648[a]	.694[a]
SDMA	.581[a]	.583[a]	.542[a]	.477[c]	.506[a]	.737[a]

TABLE 5 Correlation coefficients between NGAL concentrations/calculations and the functional renal biomarkers.

Note: ns refers to not significant ($P > .05$).
Abbreviations: FeNGAL, fractional excretion of NGAL; sCr, serum creatinine; SDMA, symmetric dimethylarginine; sNGAL, serum NGAL; sUrea, serum urea; u/sNGAL, urinary to serum NGAL ratio; uCr, urinary creatinine; uNGAL, urinary neutrophil gelatinase-associated lipocalin (NGAL); u_{norm}/sNGAL, u/sNGAL with u_{norm}NGAL; u_{norm}NGAL, normalized uNGAL; UPC, urinary protein : creatinine ratio; USG, urine specific gravity.
[a]$P \leq .001$.
[b]$.01 < P \leq .05$.
[c]$.001 < P \leq .01$.

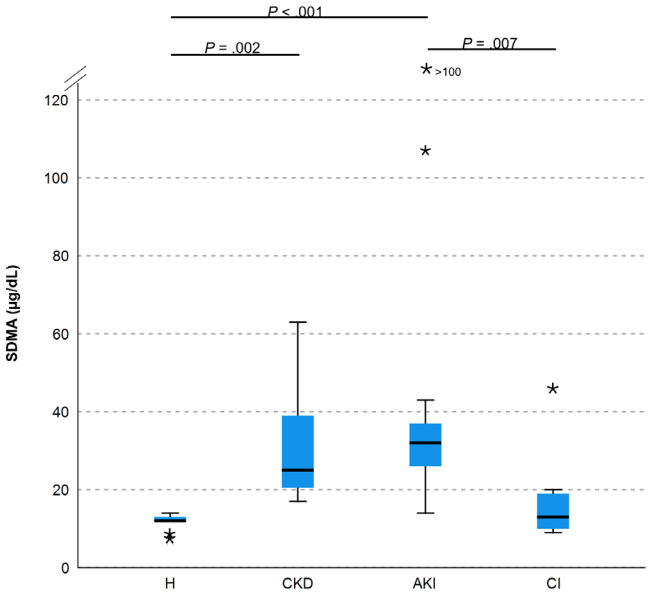

FIGURE 3 Results for SDMA in all patient groups. Bolt horizontal line: median; blue box: area between quartile 1 and 3; whiskers: upper and lower bound 1.5-3 times outside the interquartile range; asterisk: outliers (>3 times quartile 1 or 3); adjusted *P*-values are reported. AKI, acute kidney injury; CI, critically ill; CKD, chronic kidney disease; H, healthy; SDMA, symmetric dimethylarginine.

and UPC. Moderate to strong positive correlations were observed for FeNGAL with sCr, sUrea, SDMA, and UPC. Weak to moderate negative correlations were found for all NGAL variables with USG. Correlations between all NGAL variables and the functional renal biomarkers are summarized in Table 5.

3.4 | Symmetric dimethylarginine

Symmetric dimethylarginine was significantly higher in the AKI group, compared to healthy ($P < .001$) and CI ($P = .007$) dogs. All dogs with AKI (n = 11) had an SDMA >14 µg/dL, whereas 10/11 dogs with CKD and 4/10 CI dogs had an SDMA >14 µg/dL. Results are presented in Table 1 and group differences are shown in Figure 3. Strong positive correlations were found between SDMA and sCr and sUrea, whereas only a moderate negative correlation was seen for SDMA with USG.

4 | DISCUSSION

The main findings of this prospective study were that urinary cell cycle arrest biomarkers (uTIMP-2 and uIGFBP7) could be measured in canine urine and that uTIMP-2/uCr and [uTIMP-2] × [uIGFBP7]/uCr were significantly higher in dogs with AKI compared to dogs with CKD, CI, and healthy dogs. Furthermore, all NGAL variables (uNGAL, u_{norm}NGAL, sNGAL, u/sNGAL, u_{norm}/sNGAL, FeNGAL) were significantly higher in dogs with AKI compared to healthy dogs, which is compatible with previous studies that investigated urinary and plasma NGAL concentrations in dogs with renal azotemia.[28,29,32,33] In contrast to findings in people,[20] u/sNGAL, u_{norm}/sNGAL, and FeNGAL did not differentiate dogs with AKI from dogs with CKD in our study.

When exposed to either cellular stress or direct injury, TIMP-2 and IGFBP7 can be secreted by renal tubular cells to induce G1 cell cycle arrest to prevent proliferation of injured cells and to provide opportunity to repair DNA damage, so function can be restored.[6,34] Renal cell arrest usually occurs 24-48 hours before sCr increases, highlighting the important diagnostic window for early detection of AKI.[18] In human medicine, the product of uTIMP-2 and uIGFBP7 has proven to be an excellent predictor of development of AKI in CI patients or people undergoing complex surgeries.[25,34,35] Currently, 2 commercially available immunoassays, the original fluorimetric strip test "Nephrocheck" necessitating the Astute fluorimeter and the more recent "Vidas Nephrocheck," are routinely used to predict the development of (hospital-acquired) AKI in people at risk. These assays measure the absolute concentrations of uTIMP-2 and uIGFBP7 and calculate their product within 1 hour. Based on the result of these

tests, early renal damage can be detected with a sensitivity and specificity superior to other renal biomarkers.[6,7,25]

Normalized uTIMP-2 and [uTIMP-2] × [uIGFBP7]/uCr were significantly higher in dogs with AKI compared to the other diseased groups and healthy dogs. An overlap between groups was noticed, possibly indicating that the level of cellular stress was already decreasing in dogs with established AKI and that these markers have the highest diagnostic potential in the early or developing stages of AKI.[35] A correlation of the cell cycle arrest biomarkers with currently used routine kidney biomarkers was noticed but was mostly mild to moderate.[27] This was expected as the cell cycle arrest biomarkers increase as soon as cellular stress occurs, whereas the routine biomarkers are damage or functional biomarkers that change during later phases in the pathophysiological events of AKI. A small number of healthy dogs and dogs with CKD had high uTIMP-2 or uIGFBP7 values, whereas the other variables and their medical records did not reveal evidence of AKI. Cellular stress at the level of the kidneys that did not lead to development of AKI in the healthy dogs or progressive renal disease in the dogs with CKD cannot be excluded.

Neutrophil gelatinase-associated lipocalin is synthesized in the bone marrow during myelopoiesis and stored in gelatinase neutrophilic granules.[17] In addition, it can be expressed by stimulation of inflammatory mediators in several non-hematopoietic tissues such as kidney, colon, trachea, and lung epithelium.[36] Under physiological conditions, circulating NGAL is freely filtered through the renal glomeruli because of its low molecular weight and positive charge, and subsequently fully reabsorbed in the proximal tubule.[9] Consequently, no or little NGAL is expected in urine. In case of renal damage, the increase in uNGAL can be attributed to either an increased tubular expression of NGAL with direct secretion of NGAL in the urine or a decreased tubular NGAL reabsorption. The presence of uNGAL can therefore be a marker of respectively renal parenchymal damage, defective tubular reabsorption or a combination thereof.[9,37] Both conditions can but do not necessarily coexist. Urinary NGAL increases 2-3 hours after ischemic renal injury and peaks at 6 to 12 hours in CI people depending on the severity of the injury.[38-40] Because NGAL is stored in neutrophils, pyuria (independently of its origin) can be another reason for increased uNGAL values.[41,42] Apart from 1 dog with pyelonephritis in the AKI group, none of the patients had pyuria at the moment of sampling. An influence of pyuria on urinary cell cycle arrest biomarker has, to our knowledge, not been reported.

Several studies found elevated NGAL concentrations in plasma and urine of dogs with either AKI or CKD.[16,28,29,32,43-45] In human medicine, the discriminatory ability between AKI and CKD of u/sNGAL and FeNGAL was recently published, concluding that both calculations could distinguish between patients with AKI and CKD, with the highest potential for u/sNGAL.[20] The values of u/sNGAL, u_{norm}/sNGAL, and FeNGAL were higher in dogs with AKI compared to CKD in our study, although no statistical significance was reached. This might be because of a lack of power because of the rather small patient groups, but interspecies differences in underlying causes and severity of AKI and CKD also need to be considered.

Increased plasma and urinary NGAL concentrations have both proven to be valuable early diagnostic markers of acute kidney disease in CI people.[14,19,46,47] In the current study, all NGAL variables were higher in the CI dogs in comparison with the healthy dogs, corroborating previous studies. Higher sNGAL and u_{norm}NGAL values were reported for dogs with sepsis, when compared to healthy dogs.[18] A study comparing NGAL values in volume-responsive AKI and intrinsic AKI, described higher uNGAL values in dogs with inflammatory AKI than dogs with other causes of AKI.[29] Elevated NGAL concentrations in CI dogs are expected given the origin of NGAL and the inflammatory state at time of admission. Serum NGAL and FeNGAL were significantly higher in dogs with AKI compared to CI dogs, which might imply their potential value in early diagnosis of AKI in CI or hospitalized dogs. Nevertheless, more overlap of sNGAL and FeNGAL between the AKI and CI dogs was seen, compared to the overlap between dogs with AKI and healthy dogs. Possible explanations for this observation are the influence of inflammation on NGAL concentrations in CI dogs and the presence of tubular damage in some CI dogs.

Urinary excretion of any biomarker that is filtered via the glomerulus will be affected by the GFR and resultant urinary flow. Therefore, it is generally accepted that a lack of normalization can lead to falsely low biomarker concentrations in states of reduced GFR.[48] However, this interpretation is questionable in patients with acute renal disease, because urinary biomarker excretion can be affected differently in acute disease states, whereas they should be affected more similarly in chronic disease states.[49] This is a valid consideration, as creatinine excretion by kidneys is a dynamic process and normalization of urinary AKI biomarkers to uCr could overestimate acute kidney damage as a result of acute and unstable changes in creatinine clearance.[49] To date, no consensus exists in veterinary medicine whether urinary biomarkers should be normalized to uCr or not. To ensure that the significant difference between groups for the normalized values was not only influenced by difference in uCr, this variable was statistically compared between groups. Only healthy dogs had a significantly lower uCr, indicating that other group differences were because of alterations in urinary biomarker excretion.

Symmetric dimethylarginine, a commonly used renal biomarker for CKD in veterinary practice, is a by-product of protein methylation that enters the circulation after proteolysis. It is mainly excreted by the kidneys (>90%) and tubular reabsorption is absent.[50] Before the start of this study, only 1 study evaluated the value of SDMA to diagnose AKI in dogs.[30] Although SDMA was deemed a suitable renal biomarker for identifying dogs with AKI and CKD, SDMA was not significantly different between these 2 groups,[30] corroborating our findings. Because CI dogs have a higher risk to develop AKI (hospital-acquired AKI), SDMA was also measured in this group. Similar to sCr, SDMA was significantly higher in dogs with AKI compared to CI dogs, indicating that monitoring SDMA in CI dogs might have value to identify hospital-acquired AKI. Some of the CI dogs in the present study showed moderately to severely elevated SDMA concentrations. Although this might be explained by prerenal factors, the concurrent sCr elevation was less pronounced. Possible other reasons that need

to be explored are early kidney injury in these dogs or alterations in SDMA metabolism caused by the critical illness.

Having appropriate control groups is mandatory to evaluate the diagnostic potential of new biomarkers. Many studies in veterinary medicine evaluating renal biomarkers, compare dogs with AKI to healthy dogs.[2,32,51,52] However, one of the biggest challenges in veterinary practice and mainly in emergency and critical care is to determine whether CI dogs have concurrent hospital-acquired AKI. Therefore, a group of CI dogs without obvious signs of AKI was included in the current study.

This study has several limitations. The first limitation is the rather small sample size, which could have resulted in a lack of statistical power. Second, CI dogs (both at admission and during hospitalization) are at risk for hospital-acquired AKI. Although, this makes them a group of interest for renal biomarkers, AKI IRIS grade 1 cannot be fully excluded in these dogs. Third, dogs with AKI were included from IRIS grade 2 onwards because IRIS grade 1 is currently difficult to diagnose. However, the stress and damage renal biomarkers are expected to be of added value mainly in dogs with AKI grade 1. Fourth, we measured uTIMP-2 and uIGFBP7 by using canine TIMP-2 and IGFBP7 ELISA kits. As these kits were only validated for serum by the companies, limited validation of the ELISA kits for urine was performed. Results of the in-house validation revealed acceptable precision and acceptable to poor accuracy (see Supplemental Information and Table S1). No quality control samples were used and because of a limited amount of available ELISA kits, samples were not analyzed in duplicate. Therefore, absolute values should be interpreted with caution. For TIMP-2, values below the lowest standard point were reported and included for statistical analysis. The performance of this ELISA kit below 20 pg/mL needs to be evaluated before further studies. Finally, the combination of antibodies that is used in most NGAL immunoassays (including the ELISA that was used in this study) does not distinguish between molecular forms of NGAL that are produced by the tubular cells from those which are synthesized or released by other sources (mainly neutrophils).[32,41,53] Both in human and veterinary medicine, the NGAL molecule exists in at least 3 different forms in blood and urine, as a 25 kDa monomer, a 45 kDa homodimer, and a 135 kDa heterodimer where the protein is covalently bonded to matrix metalloproteinase.[41,54,55] The monomer is predominantly released by tubular cells, whereas the homodimer is mostly expressed by neutrophils.[53] Measuring only the monomer, namely the kidney-specific NGAL (eg, by using sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Western blotting[41]) might lead to better group differentiation and increase specificity of NGAL measurements in future research. However, this procedure is even more time-consuming than the currently used canine NGAL ELISA kit.

In conclusion, uTIMP-2/uCr and [uTIMP-2] × [uIGFBP7]/uCr were the only renal biomarkers that were significantly higher in dogs with AKI compared to dogs with CKD, CI, and healthy dogs. Further in-depth evaluation whether these cell cycle arrest biomarkers can identify AKI grade 1 in CI dogs and whether monitoring of these markers over time in dogs at risk of AKI provides added diagnostic value is therefore warranted. Additionally, sNGAL, FeNGAL, and SDMA measured at admission were also significantly higher in dogs with AKI than CI dogs and could be of value in early detection of hospital-acquired AKI. Follow-up studies in CI dogs are recommended to further explore this.

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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

Approval granted by the local ethical committee of the Faculty of Veterinary Medicine, Ghent University, Belgium and the deontological committee of the Belgian Federal Agency for the Safety of the Food Chain (EC 2020/067).

HUMAN ETHICS APPROVAL DECLARATION

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

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SUPPORTING INFORMATION

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

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