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Received: 14 May 2020 | Accepted: 4 February 2021

DOI: 10.1111/jvim.16074

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Liver-type fatty acid-binding protein and neutrophil gelatinase-associated lipocalin in cats with chronic kidney disease and hyperthyroidism

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Funding information
IDEXX Laboratories; Mahidol University

Abstract

Background: Liver-type fatty acid-binding protein (L-FABP) and neutrophil gelatinase-associated lipocalin (NGAL) are candidate biomarkers for the detection of early chronic kidney disease (CKD) in cats.

Objective: To evaluate urinary and serum L-FABP and NGAL concentrations in CKD cats and in hyperthyroid cats before and after radioiodine (^{131}I) treatment.

Animals: Nine CKD cats, 45 healthy cats and hyperthyroid cats at 3 time points including before (T0, n = 49), 1 month (T1, n = 49), and 11 to 29 months after (T2, n = 26) ^{131}I treatment.

Methods: Cross-sectional and longitudinal study. Serum L-FABP (sL-FABP), serum NGAL (sNGAL), urinary L-FABP (uL-FABP), and urinary NGAL (uNGAL) were compared between the 3 groups and between hyperthyroid cats before and after treatment. Data are reported as median (min-max).

Results: CKD cats had significantly higher sL-FABP (13.50 [3.40-75.60] ng/ml) and uL-FABP/Cr (4.90 [0.97-2139.44] μg/g) than healthy cats (4.25 [1.34-23.25] ng/ml; P = .01 and 0.46 [0.18-9.13] μg/g; P < .001, respectively). Hyperthyroid cats at T0

Abbreviations: ^{131}I, radioactive iodine; CKD, chronic kidney disease; GFR, glomerular filtration rate; IRIS, international Renal Interest Society; L-FABP, liver-type fatty acid-binding protein; LOD, lower limit of detection; NGAL, neutrophil gelatinase-associated lipocalin; pNGAL, plasma NGAL; RI, reference interval; ROC, receiver operator characteristic; SBP, systolic blood pressure; sCr, serum creatinine; sL-FABP, serum L-FABP; sNGAL, serum NGAL; sTSH, serum thyroid-stimulating hormone; sTT4, serum total thyroxine; T0, before treatment time point; T1, 1 month after treatment time point; T2, 11 to 29 months after treatment time points; uL-FABP, urinary L-FABP; uL-FABP/Cr, urinary L-FABP-to-creatinine ratio; uNGAL, urinary NGAL; uNGAL/Cr, urinary NGAL-to-creatinine ratio; UPC, urinary protein-to-creatinine ratio; USG, urine specific gravity.

had significantly higher uL-FABP/Cr (0.94 [0.15-896.00] μg/g) than healthy cats ($P < .001$), thereafter uL-FABP/Cr significantly decreased at T2 (0.54 [0.10-76.41] μg/g, $P = .002$). For the detection of CKD, uL-FABP/Cr had 100% (95% confidence interval [CI], 66.4-100.0) sensitivity and 93.2% (95% CI, 81.3-98.6) specificity. There were no significant differences in sNGAL and uNGAL/Cr between the 3 groups.

Conclusions and Clinical Importance: L-FABP, but not NGAL, is a potential biomarker for the detection of early CKD in cats. Utility of uL-FABP to predict azotemia after treatment in hyperthyroid cats remains unknown.

KEYWORDS
biomarkers, cats, CKD, hyperthyroid, L-FABP, NGAL

1 | INTRODUCTION

Chronic kidney disease (CKD) in cats is routinely diagnosed by combined presence of azotemia and inappropriate urine specific gravity (USG).[1,2] Glomerular filtration rate (GFR) testing is the gold standard for CKD diagnosis, but it is impractical in routine clinical practice.[3] Traditional renal biomarkers such as serum creatinine (sCr) only detect renal dysfunction at a late stage of disease progression.[4] Early detection of CKD might enable early therapeutic intervention to delay CKD progression in cats. Hence, the limitations of these biomarkers have driven the search for other biomarkers that are potential for early detection of CKD.[5]

Hyperthyroidism affects 4% to 11% of elderly cats,[6] 30% to 50% of which develop CKD.[7,8] Concurrent CKD can be masked by hyperthyroidism, and post-treatment azotemia develops in 15% to 40% of hyperthyroid cats.[9-11] The evaluation of CKD in hyperthyroidism is particularly challenging, because thyrotoxicosis increases the GFR and induces loss of muscle mass,[12-14] consequently lowering sCr concentrations.[14] Specifically in hyperthyroid cats, serum symmetric dimethylarginine has only low sensitivity for the detection of post-treatment azotemia and did not accurately reflect GFR.[15,16] This is an additional reason to search for alternative early renal biomarkers that are also useful in hyperthyroid.

Liver-type fatty acid-binding protein (L-FABP) is expressed in the liver and renal proximal tubular cells. Freely filtered L-FABP from circulation is normally reabsorbed in the proximal tubules.[17,18] Stress on the renal tubules increases uL-FABP levels.[19] Urinary L-FABP appears to be useful for detecting early-stage renal disease and has prognostic value and utility in monitoring CKD progression in humans.[20-24] Katayama et al suggest that urinary L-FABP-to-creatinine ratio (uL-FABP/Cr) may be a potential biomarker to predict early renal injury.[25,26] To corroborate these findings and to evaluate whether L-FABP has potential as an early CKD biomarker in hyperthyroid cats, longitudinal study of L-FABP in hyperthyroid cats along with comparison between hyperthyroid cats, healthy cats, and CKD cats are warranted.

Neutrophil gelatinase-associated lipocalin (NGAL) is expressed by neutrophils and renal tubular cells during injury and inflammation.[27,28] Freely pass through the glomerulus, NGAL is well reabsorbed in the proximal tubules.[29] NGAL is upregulated because of increased renal tubular secretion and decreased reabsorption by injured proximal tubules.[30,31] Both serum and urinary NGAL (sNGAL and uNGAL) are used to detect acute kidney injury and CKD in humans and dogs.[32-42] CKD cats have a higher uNGAL/Cr than do healthy cats.[43] Currently, studies of uNGAL as a renal biomarker in CKD cats and in hyperthyroid cats are lacking.

After validation of 2 commercial assays for feline L-FABP and NGAL, the first objective of this study was to evaluate both serum and urine L-FABP and NGAL concentrations in CKD cats and in hyperthyroidism. Second, this study aimed to evaluate both renal biomarkers in hyperthyroid cats before and after radioactive iodine (^{131}I) treatment.

2 | MATERIALS AND METHODS

2.1 | Study population

For this cross-sectional study, frozen (−80°C) serum and urine samples obtained during previous studies between October 2015 and November 2017 from healthy control cats, CKD cats, and hyperthyroid cats were analyzed.[16,44,45] Additionally, frozen serum and urine samples from 2 hyperthyroid cats were included.

For the longitudinal study, serum and urine samples from the same population of hyperthyroid cats were evaluated at 3 time points; before ^{131}I treatment (T0); 1 month (T1); and 11 to 29 months after ^{131}I treatment (T2). These samples were collected between 2015 and 2019 and frozen at −80°C until batch analysis in 2019.[16,45]

Adult cats were considered healthy based on absence of clinically relevant abnormalities in the history, physical examination, CBC, serum biochemistry, urinalysis, thoracic radiographs, and abdominal ultrasound examination.

Cats with CKD had clinical signs compatible with CKD, increased sCr (>2.3 mg/dL, reference interval [RI] of IDEXX Laboratories) and USG <1.035.[46] Exclusion criteria were concurrent systemic disease, hyperthyroidism, or prerenal and postrenal azotemia. CKD cats receiving drugs or supplements other than phosphate binders withdrew these treatments at least 14 days before enrollment. CKD cats were

classified into 4 stages according to the International Renal Interest Society (IRIS) guidelines.[1]

Hyperthyroidism was diagnosed at T0 based on compatible clinical signs, increased serum total thyroxine (sTT4) concentration, or increased thyroid uptake of pertechnetate. In cats previously treated with antithyroid drugs (methimazole), the drug was stopped 10 days before ^{131}I treatment. Cats were treated with adapted doses of ^{131}I injected IV (mean dose 142.6 MBq [3.9 mCi]; range, 67.7-455.1 MBq [1.8-12.3 mCi]) after the first examination and sample collection (T0).[47] Hyperthyroid cats were excluded if they had pre-existing azotemia (sCr >2.3 mg/dL), any evidence of concurrent systemic disease, or use of any medication except for antithyroid medication within the 2 weeks before enrollment. At T1 and T2, cats' thyroid status was determined based on the combination of sTT4 (RI 0.8-4.7 μg/dL) and serum thyroid-stimulating hormone (sTSH) (RI ≤0.3 ng/mL) concentrations as euthyroid (both sTT4 and sTSH within RI), subclinically hypothyroid (sTT4 within RI and sTSH above RI), hypothyroid (sTT4 below RI and sTSH above RI), and hyperthyroid (sTT4 above RI and undetectable sTSH).[48] Cats at T1 or T2 with sCr above >2.3 mg/dL, USG <1.035, and no evidence of prerenal or postrenal azotemia were defined as having post-treatment azotemia.

The Faculty of Veterinary Medicine, Ghent University, Belgium local ethical committee (EC 2015/68 and EC 2017/72) approved the study protocol. Participating owners provided written informed consent.

2.2 | Procedures

All cats underwent a thorough examination including medical history, complete physical examination, body weight measurement, body condition score (9-point scale), systolic blood pressure (SBP) using Doppler ultrasound technique, and thyroid gland palpation. Standard 2-view thoracic radiographs (lateral and ventrodorsal projections) and complete abdominal ultrasound examinations were performed to screen for concurrent diseases in all cats at baseline. Echocardiography was also performed in hyperthyroid cats at T0 to check for the presence of concurrent heart disease.

A subset of hyperthyroid cats had GFR measurements performed by exogenous plasma Cr clearance testing by a protocol previously described by our research group at 3 time points (T0, T1, and T2).[49] The GFR cut-off values were defined as normal GFR value (≥1.9 mL/minute/kg), borderline-low value (1.4-1.8 mL/minute/kg) and low GFR (< 1.4 mL/minute/kg) based on previous data from our research group.[50]

2.3 | Blood and urine samples

Blood samples were taken by jugular venipuncture. Blood work consisted of complete blood count and serum biochemistry including sCr (IDEXX laboratories, Inc., Westbrook, Maine). Cats with CKD and healthy control cats >6 years of age underwent sTT4 measurement. Serums TT4 and sTSH were measured in hyperthyroid cats at all time points (IDEXX Laboratories). Residual serum was stored at −80°C for 1 to 4 years until batch analysis of sL-FABP and sNGAL.

Urine samples taken by cystocentesis were sent to IDEXX Laboratories for urinalysis. Urinalysis consisted of dipstick chemistry with IDEXX UA Strips (IDEXX Laboratories, Westbrook, Maine), sediment analysis, urinary protein-to-creatinine ratio (UPC), and USG using refractometry in all cats and also included bacterial culture in CKD cats and hyperthyroid cats at T0. Residual urine was stored at −80°C for 1 to 4 years until batch analysis of uL-FABP and uNGAL.

2.4 | Analytical methods

Serum and urine creatinine concentrations were determined by an enzymatic colorimetric method based on Jaffe's method using picric acid as a reagent. Urinary protein concentrations were determined by a colorimetric assay, the pyrogallol red molybdate method. All values were obtained by IDEXX Laboratories using a Beckman Coulter AU Analyzer (Beckman Coulter, Brea, California). Serum TT4 and sTSH were measured with a Microgenics DRI TT4 enzyme immunoassay (Microgenics Corporation, Freemont, California)[51] and with an Immulite canine TSH chemiluminescent enzyme immunoassay (Siemens Healthcare Diagnostics Products, Tarrytown, New York), respectively.[52,53]

Concentrations of sL-FABP and uL-FABP were measured using a commercial feline L-FABP ELISA kit (CMIC Holding, Tokyo, Japan) using an anti-human L-FABP antibody. Concentrations of sNGAL and uNGAL were determined using a commercial feline NGAL sandwich ELISA kit (MyBiosource, San Diego, California) using an antihuman NGAL antibody. The urine samples were centrifuged at 1000 g for 20 minutes before analysis. The in-house validation report of the assays for L-FABP and NGAL in feline samples has been added as Data S1, Table S1, and Figure S1. The lower limit of detection (LOD) was 2.68 ng/mL for L-FABP assay and 3.10 ng/mL for NGAL assay in feline serum and urine samples, respectively. Therefore, before running statistical analysis, samples with an L-FABP concentration < 2.68 ng/mL were assigned the arbitrary value of 1.34 ng/mL, while samples assayed with an NGAL concentration < 3.10 ng/mL were assigned the arbitrary value 1.55 ng/mL.

2.5 | Statistical analysis

Statistical analyzes were performed using R version 3.6.3 (2020, The R Foundation for Statistical Computing). Most variables were not normally distributed according to the Shapiro-Wilks test.

For the cross-sectional study, the overall analysis was based on the Kruskal Wallis test and pairwise comparisons on the Wilcoxon rank sum test by Bonferroni's method to adjust for multiple comparisons, that is, testing each of the 3 pairwise comparisons at a significance level of .05/3. Spearman's correlations were assessed between both biomarkers (L-FABP and NGAL) and 2 other renal variables (sCr

and UPC) and between serum L-FABP (sL-FABP) or NGAL and urine L-FABP/Cr or NGAL/Cr, respectively, in the group of CKD cats and healthy cats. The sensitivity and specificity of L-FABP and NGAL for the detection of azotemic CKD in CKD cats and healthy cats were obtained by receiver operating characteristic (ROC) curve analysis.

For the longitudinal study, all variables were compared between time points (T0, T1, and T2) by pairwise comparisons on the Wilcoxon signed-rank test using Bonferroni's method to adjust for multiple comparisons, testing each of the 3 pairwise comparisons at a significance level of .05/3. Correlations between both urinary biomarkers (uL-FABP/Cr or uNGAL/Cr) and other thyroid and renal variables (sTT4, sCr, GFR, and UPC) and between sL-FABP or NGAL and urine L-FABP/Cr or NGAL/Cr, respectively, were determined in hyperthyroid cats at different time points by Spearman's correlation.

3 | RESULTS

3.1 | Study population

One hundred and nine cats (10 CKD cats, 52 hyperthyroid cats, and 47 healthy cats) were initially enrolled in the study. Samples from 6 cats were excluded, as 1 CKD cat was also hyperthyroid, 3 hyperthyroid cats had concurrent systemic disease (second-degree AV block type 2, a liver mass, and CKD), and 2 healthy cats had liver mass and bilateral small kidneys. The CKD group consisted of 2 stage 2 cats, 6 stage 3 cats, and 1 stage 4 cat based on the IRIS staging system. The remaining 49 hyperthyroid cats were presented at both T0 and T1, and 26 of them were also presented at T2. The other 23 cats were lost to follow-up at T2 because of owner non-compliance (n = 21) or because of death before assessment at T2 (n = 2).

In the CKD group, 4 cats were domestic short-hair or long-hair cats, 2 were Ragdolls cats, 2 were Bengal cats, and 1 was a British Shorthair. Represented breeds in the hyperthyroid group included 46 domestic short-hair or long-hair cats and 1 British Shorthair, Chartreux, and Norwegian Forest cat. The healthy group comprised 41 domestic short-hair or long-hair cats, 2 Ragdolls, and 1 British Shorthair cat.

For the cross-sectional study, selected clinical variables and routine kidney variables of the study population are presented as descriptive statistics in Table 1.

For the longitudinal study, selected variables at the 3 time points are presented in Table 2. Forty-one cats were euthyroid, 4 cats were subclinically hypothyroid, 1 cat was hypothyroid, 1 cat remained hyperthyroid, and 2 cats had uncertain thyroid status at T1 because of the missing sTSH concentrations. At T2, there were 18 euthyroid cats, 7 subclinically hypothyroid cats, and 1 iatrogenic hyperthyroid cat because of levothyroxine overdose. All 49 cats were nonazotemic at both T0 and T1. Only 2 euthyroid cats developed post-treatment azotemia (IRIS stages 2 and 4) at T2. The GFR measurement was performed in 11 hyperthyroid cats at 3 time points, and 1 cat at 2 time points (T0 and T1). All 12 cats had normal GFR at T0. At T1, borderline low GFR was present in 2 cats and low GFR was presented in 1 cat. At T2, 5 of 11 cats had borderline low GFR. None of the cats with GFR measurements were azotemic either at T1 or T2.

3.2 | Comparison of L-FABP and NGAL between CKD cats, hyperthyroid cats, and healthy cats

Serum L-FABP concentrations were measured in all CKD (9) and healthy cats (45) and in 40 of 49 hyperthyroid cats. Serum NGAL (sNGAL) concentrations were evaluated in 38 of 49 hyperthyroid cats. The concentrations of uL-FABP and uNGAL were measured in 44 of 45 healthy cats. All missing measurements were related to inadequate remaining sample volumes.

There were significant differences in sL-FABP and uL-FABP/Cr measurements between groups (Figure 1). Cats with CKD had significantly higher sL-FABP and uL-FABP/Cr values than healthy cats. The urinary L-FABP concentration was <LOD in 3 (33%) of 9 CKD cats, 9 (18%) of 49 hyperthyroid cats, and in 41 (93%) of 44 healthy cats. The 6 CKD cats with detectable uL-FABP had IRIS stage 2 to 4 CKD, while the remaining CKD cats with uL-FABP <LOD all had stage 3.

In hyperthyroid cats, the uL-FABP/Cr was significantly higher than in healthy cats There were no significant differences in sL-FABP

TABLE 1 Descriptive statistics of selected clinical and laboratory variables in cats with CKD, hyperthyroid cats, and healthy cats

	CKD	Hyperthyroid	Healthy
Variables			
Sex (male/total)	6/9	24/49	20/45
Age (years)	6[a] [5, 9]	12[b] [10, 14]	6[a] [3, 8]
Body weight (kg)	3.7 [3.2,5.0]	3.7 [3.4-4.3]	4.0 [3.4-4.9]
SBP (mmHg)	145 [140, 151]	155 [140, 180]	146 [130, 158]
sCr (mg/dL)	3.43[a] [2.76, 4.07]	0.69[b] [0.57, 0.97]	1.23[c] [1.06, 1.37]
USG	1.019[a] [1.017, 1.020]	1.050[b] [1.036, 1.050]	1.049[b] [1.042, 1.054]
UPC	0.2[a,b] [.1, .8]	0.5[a,] [0.4, 0.8]	0.1[b] [0.1, 0.2]

Note: Data are presented as medians [25th and 75th percentiles], except for sex (proportions). Medians in a row with different superscripts (a,b,c) differ significantly.
Abbreviations: CKD, chronic kidney disease; SBP, systolic blood pressure; sCr, serum creatinine; UPC, urinary protein-to-creatinine ratio; USG, urine specific gravity.

TABLE 2 Selected clinical and laboratory variables of hyperthyroid cats before (T0), 1 month (T1), and 11 to 29 months after (T2) ^{131}I treatment

	T0	T1	T2
Variables			
Body weight (kg)	3.7[a] [3.4, 4.3]	4.1[b] [3.5, 4.6]	4.1[c] [3.6, 4.9]
SBP (mmHg)	155 [140, 180]	165 [140, 180]	170 [150, 182]
sTT4 (μg/dL)	8.1[a] [6.5, 12]	0.9[b] [0.7, 1.3]	1.7[c] [1.4, 2.0]
sTSH (ng/mL)	<0.03[a] [<0.03, <0.03]	0.09[b] [<0.03, 0.15]	0.17[c] [0.07, 0.35]
sCr (mg/dL)	0.69[a] [0.57, 0.97]	1.29[b] [0.98, 1.50]	1.54[c] [1.35, 1.77]
USG	>1.050[a] [1.035, >1.050]	1.042[b] [1.028, >1.050]	1.036[b] [1.021, 1.050]
UPC	0.5[a] [0.4, 0.8]	0.2[b] [0.1, 0.3]	0.2[b] [0.1, 0.3]
GFR (mL/kg/minute)	3.2[a] [2.8, 4.2]	2.1[b] [1.9,2.3]	2.0[b] [1.6, 2.5]

Note: Data are presented as median [25th and 75th percentiles]. Medians in a row with different superscripts (a,b,c) differ significantly.
Abbreviations: GFR, glomerular filtration rate; SBP, systolic blood pressure; sCr, serum creatinine; sTSH, serum thyroid stimulating hormone; sTT4, serum total thyroxine; UPC, urinary protein-to-creatinine ratio; USG, urine specific gravity.

concentrations between hyperthyroid and CKD cats. No significant differences in sNGAL and uNGAL/Cr were observed between groups (Figure 2).

3.3 | Receiver operating characteristic analysis of L-FABP and NGAL for the detection of azotemic CKD

Receiver operating characteristic analysis illustrated the ability of L-FABP and NGAL to distinguish azotemic CKD cats from healthy cats.

For sL-FABP, a cutoff of ≥13.50 ng/mL had 55.6% [95% CI, 21.2-86.3] sensitivity and 88.9% [95% CI, 76.0-96.3] specificity. Five (11%) of 45 healthy cats showed sL-FABP concentrations greater than the cutoff. For uL-FABP/Cr, a cutoff of ≥0.97 μg/g had a 100% [95% CI, 66.4-100.0] sensitivity and 93.2% [95% CI, 81.3-98.6] specificity (Figure 3). Three of 44 healthy cats had uL-FABP/Crs greater than the cutoff.

The cutoff for sNGAL was ≥48.38 ng/mL, which had a sensitivity of 33.3% [95% CI, 7.48-70.1] and a specificity of 84.4% [95% CI, 70.5-93.5]. The sensitivity and specificity were 55.6% [95% CI, 21.2-86.3] and 93.2% [95% CI, 81.3-98.6] for uNGAL/Cr with a cutoff of ≥7.39 μg/g. Three of 44 healthy cats had uNGAL/Crs greater than the cutoff.

3.4 | Comparison of L-FABP and NGAL in hyperthyroid cats before and after ^{131}I treatment

Serum L-FABP concentration did not significantly differ between T0 and T2 and between T1 and T2 (Figure 4A). Urinary L-FABP/Cr significantly decreased at T2 compared to T0 while it did not significantly differ between T0 and T1 and between T1 and T2 (Figure 4B). At T0, uL-FABP concentration was significantly different from T1 ($P < .001$) and T2 ($P = .003$). The concentration of uL-FABP at the different time points is shown in Figure 5A. This concentration was less than LOD in

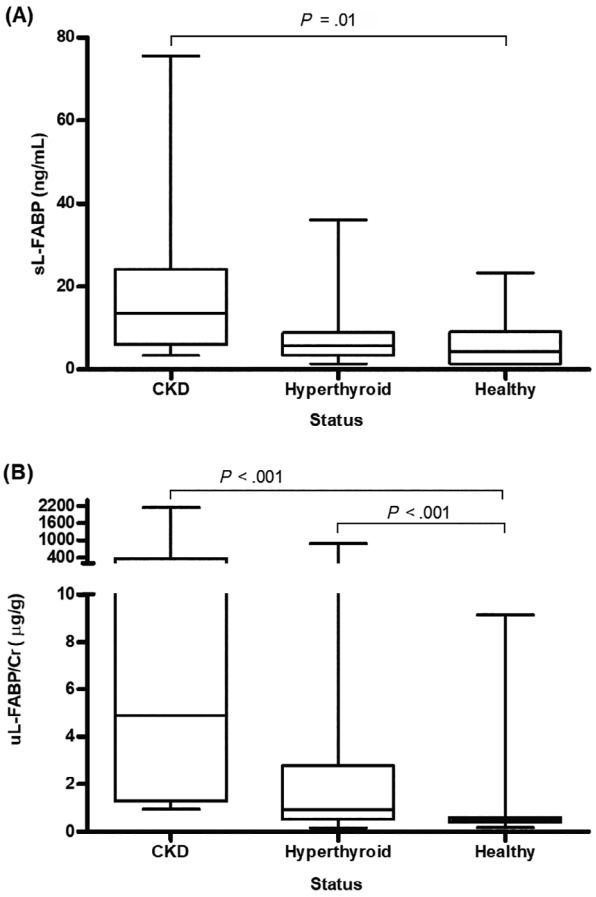

FIGURE 1 Box and whisker plots showing L-FABP in CKD cats, hyperthyroid cats, and healthy cats. A, sL-FABP concentrations in CKD cats (n = 9), hyperthyroid cats (n = 40), and healthy cats (n = 45) and their significant differences. B, uL-FABP/Cr in CKD cats (n = 9), hyperthyroid cats (n = 49), and healthy cats (n = 44) and their significant differences. Whiskers represent the min and max values. CKD, chronic kidney disease; L-FABP, liver-type fatty acid-binding protein; sL-FABP, serum L-FABP; uL-FABP/Cr, urinary L-FABP-to-creatinine ratio

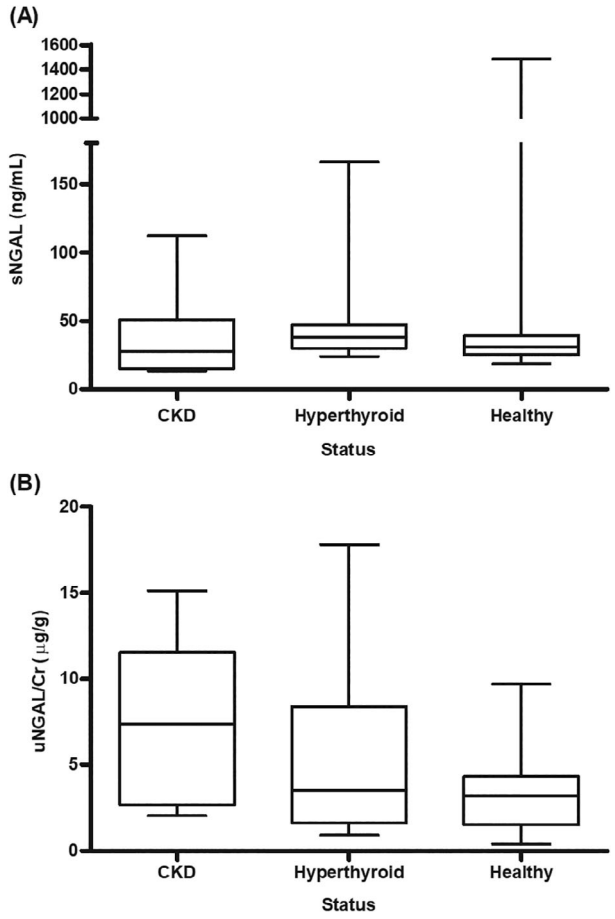

FIGURE 2 Box and whisker plots showing NGAL in CKD cats, hyperthyroid cats, and healthy cats. A, sNGAL concentrations in CKD cats (n = 9), hyperthyroid cats (n = 38), and healthy cats (n = 45) and their significant differences. B, uNGAL/Cr in CKD cats (n = 9), hyperthyroid cats (n = 49), and healthy cats (n = 44). Whiskers represent the min and max values. CKD, chronic kidney disease; NGAL, neutrophil gelatinase-associated lipocalin; sNGAL, serum NGAL; uNGAL/Cr, urinary NGAL-to-creatinine ratio

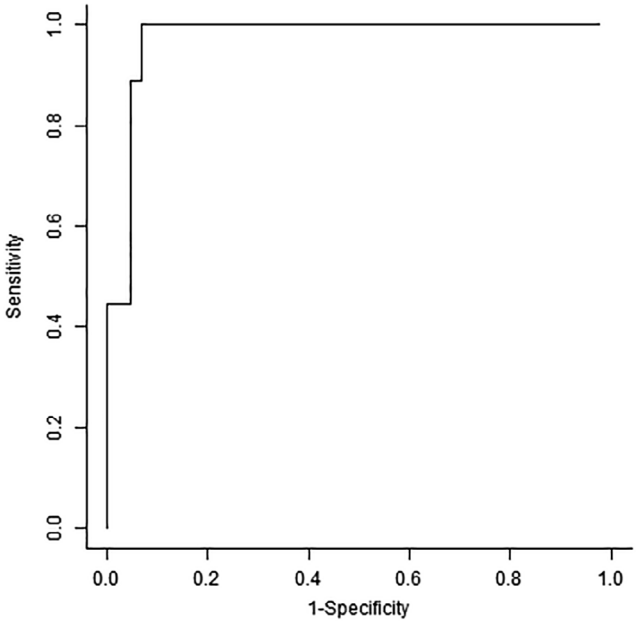

FIGURE 3 Receiver operating characteristic curve displaying the sensitivity and specificity of urinary L-FABP-to-creatinine ratio as a diagnostic test for azotemic chronic kidney disease (CKD) in cats with azotemic CKD (n = 9) and healthy cats (n = 45)

32 (65%) of 49 cats at T0, in 47 (96%) of 49 cats at T1, and 23 (89%) of 26 cats at T2.

Serum NGAL concentration significantly decreased at T2 compared to T0 and T1. There was no significant different in sNGAL concentration between T0 and T1 (Figure 6A). Urinary NGAL/Cr did not significantly change between time points (Figure 6B). The concentration of uNGAL was not significantly different between time points. The concentration of uL-FABP at the different time points is presented in Figure 5B.

Comparison of both renal biomarkers (L-FABP and NGAL) from T0 to T2 between the subgroup of cats with different GFR status at T2 is described in Table S2.

3.5 | Correlations between L-FABP and NGAL and routine renal variables

Spearman's correlations between both renal biomarkers (L-FABP and NGAL) and 2 routine renal variables, namely sCr and UPC, were assessed in CKD cats and in healthy cats. The data are presented in Table 3.

Significant correlations between sL-FABP and sCr and between sL-FABP and UPC were weak and moderate, respectively. Urinary L-FABP/Cr was weakly and significantly correlated with both sCr and UPC. There were no significant correlations between sNGAL and both sCr and UPC. The correlation between uNGAL/Cr and sCr was not significant, whereas the correlation between uNGAL/Cr and UPC was weak and significant.

In hyperthyroid cats before and after ^{131}I treatment, Spearman's correlations between both renal biomarkers (L-FABP and NGAL) and selected variables including sCr, UPC, sTT4, and GFR at different time points were calculated and reported in Table 4.

Urinary LFABP/Cr significantly correlated with UPC at all time points with a moderate correlation at both T0 and T2 and with a weak correlation at T1. A moderate and significant correlation was observed between GFR and uL-FABP/Cr at T0. The correlations between uNGAL/Cr and UPC were significant at T1 and T2 with weak and strong correlations, respectively. At all time points, sL-FABP and sNGAL did not correlate with all selected variables, uL-FABP/Cr did not correlate with sCr and sTT4, and uNGAL/Cr did not correlate with sCr, GFR, and sTT4.

3.6 | Correlations between serum and urinary concentrations for L-FABP and NGAL

Spearman's correlation analyzes demonstrated that log sL-FABP concentrations were significantly correlated with the log uL-FABP/Cr

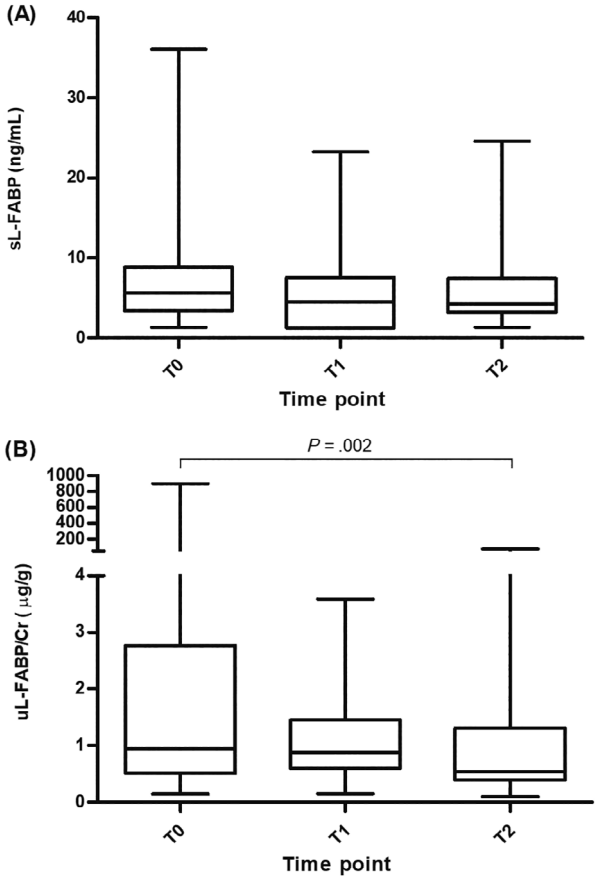

FIGURE 4 Box and whisker plots showing L-FABP in hyperthyroid cats before (T0), 1 month (T1), and 11 to 29 months after (T2) ^{131}I treatment. A, sL-FABP concentrations in cats at T0 (n = 40), T1 (n = 42), and T2 (n = 22). B, uL-FABP/Cr in cats at T0 (n = 49), T1 (n = 49), and T2 (n = 26). Whiskers represent the min and max values. L-FABP, liver-type fatty acid-binding protein; sL-FABP, serum L-FABP; uL-FABP/Cr, urinary L-FABP-to-creatinine ratio

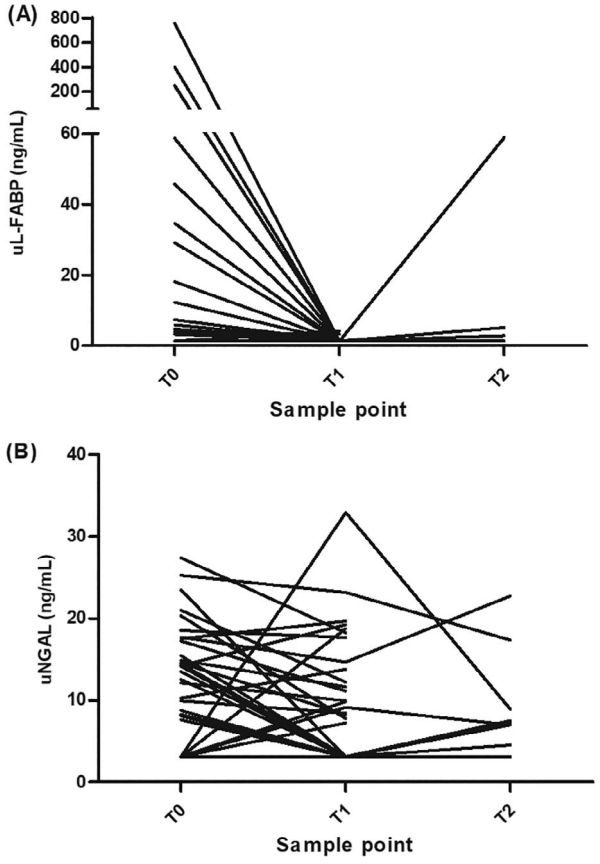

FIGURE 5 The concentrations of (A) uL-FABP and (B) uNGAL in hyperthyroid cats before (T0, n = 49), 1 month (T1, n = 49), and 11 to 29 months after (T2, n = 26) ^{131}I treatment. L-FABP, liver-type fatty acid-binding protein; NGAL, neutrophil gelatinase-associated lipocalin; uL-FABP/Cr, urinary L-FABP-to-creatinine ratio; uNGAL/Cr, urinary NGAL-to-creatinine ratio

values in CKD cats and healthy cats (ρ = .31; P = .03; Figure 7A). There was no significant correlation between log sNGAL and log uNGAL/Cr in CKD cats and healthy cats (ρ = .18; P = .21; Figure 7B).

In hyperthyroid cats before and after ^{131}I treatment, Spearman's correlations between sL-FABP and uL-FABP/Cr were not significant in hyperthyroid cats at all time points. There were no significant correlations between sNGAL and uNGAL/Cr in cats at all time points.

4 | DISCUSSION

In the present study, we investigated the diagnostic utility of L-FABP for detecting azotemic CKD in cats. Furthermore, this is the first study to evaluate both serum and urinary L-FABP as well as NGAL in hyperthyroid cats. The main findings of this study are: (a) CKD cats have significantly higher sL-FABP and uL-FABP values than healthy cats; (b) most healthy cats have a uL-FABP concentration below the LOD; (c) sL-FABP significantly correlated with uL-FABP/Cr in CKD cats and in healthy cats; (d) both sNGAL and uNGAL values are not different between CKD, hyperthyroid, and healthy cats; (e) ROC analysis indicated that uL-FABP/Cr was superior to sL-FABP, sNGAL, and uNGAL/Cr for differentiating azotemic CKD from healthy cats; (f) hyperthyroid cats have higher uL-FABP values compared to healthy cats and these values normalize after restoring euthyroidism.

CKD cats had significantly higher s L-FABP concentration than healthy cats. This observation is compatible with findings in human studies. Indeed, significantly higher sL-FABP concentrations are detected in patients with end-stage renal disease compared to healthy volunteers.[54] In addition, the significant correlation between sL-FABP and sCr we observed is in line with findings described in human CKD patients.[55] Using a cutoff of 13.5 ng/mL, an increased sL-FABP concentration was present in half of the CKD cats with a specificity approximating 90%. An unexpected finding was that sL-FABP in CKD cats varied widely regardless of IRIS stage. Although careful interpretation is warranted considering the low number of CKD cats included in the current study, the explanation is not obvious. Little is known about the mechanism causing an increased sL-FABP concentration in patients with CKD. In healthy individuals, circulatory L-FABP mainly originates from the liver and is increased in patients with liver

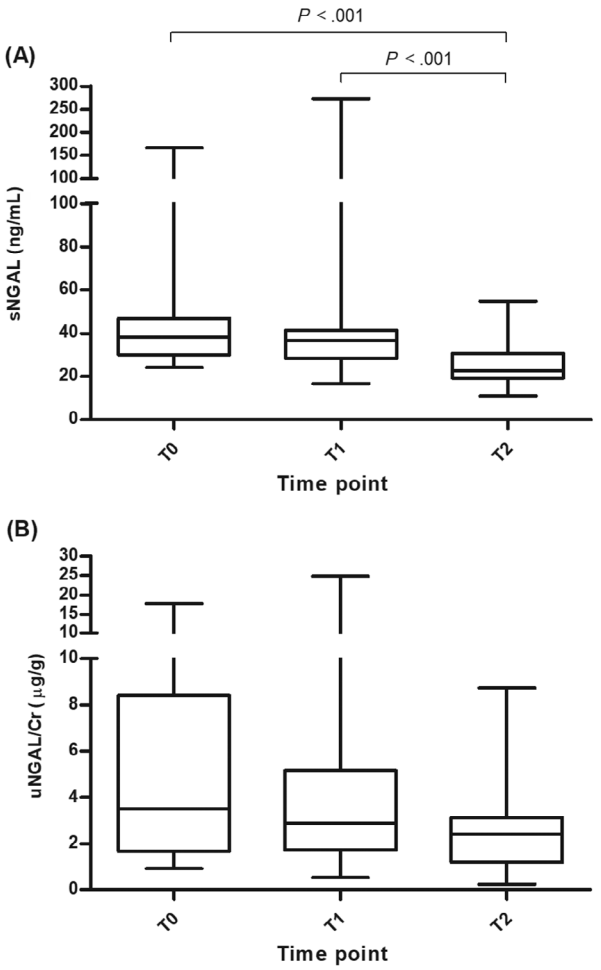

FIGURE 6 Box and whisker plots showing NGAL in hyperthyroid cats before (T0), 1 month (T1), and 11 to 29 months after (T2) ^{131}I treatment. A, NGAL concentrations in cats at T0 (n = 38), T1 (n = 41), and T2 (n = 22). B, uNGAL/Cr in cats at T0 (n = 49), T1 (n = 49), and T2 (n = 26). Whiskers represent the min and max values. NGAL, neutrophil gelatinase-associated lipocalin; sNGAL, serum NGAL; uNGAL/Cr, urinary NGAL-to-creatinine ratio

disease.[55] Serum L-FABP is filtered through the kidney, so its accumulation may be caused by a decreased GFR in patients with CKD. If this hypothesis is true, sL-FABP concentrations would be expected to be associated with IRIS stage in CKD cats. Another theory is that upon tubulointerstitial injury, uL-FABP may be reabsorbed by the still intact proximal tubules, thereby increasing sL-FABP.[56] Cats with CKD may have different degrees of tubulointerstitial damage, so renal biopsy would be beneficial to further evaluate L-FABP expression in relation to the renal pathology present in those cats.

Five of the 45 healthy cats showed sL-FABP concentrations greater than the cutoff. Concurrent (liver) disease seems unlikely, as these cats were thoroughly screened at inclusion. None of them became azotemic during 7 months of follow-up, suggesting that early CKD is unlikely in these 5 cats. Nevertheless, whether or not the increased sL-FABP in these non-azotemic cats did or did not originate

TABLE 3 Spearman's correlations (ρ) between L-FABP and NGAL values and routine renal variables in cats with CKD and healthy cats (n = 54)

	sCr		UPC	
Variables	ρ	P	ρ	P
sL-FABP	.30	.03	.47	< .001
uL-FABP/Cr	.28	.06	.35	.01
sNGAL	−.03	.86	.15	.27
uNGAL/Cr	.08	.57	.29	.03

Abbreviations: CKD, chronic kidney disease; L-FABP, liver-type fatty acid-binding protein; NGAL, neutrophil gelatinase-associated lipocalin; sCr, serum creatinine; sL-FABP, serum L-FABP; sNGAL, serum NGAL; uL-FABP/Cr, urinary L-FABP-to-creatinine ratio; uNGAL/Cr, urinary NGAL-to-creatinine ratio; UPC, urinary protein-to-creatinine ratio.

from renal injury is impossible to determine without renal histopathological assessment.

Urinary L-FABP/Cr was significantly increased in CKD cats compared to healthy cats. This outcome is comparable to human studies showing significant increases in uL-FABP/Cr in patients with CKD.[21,24,55,57] Six (67%) of 9 CKD cats had measurable uL-FABP concentrations, corroborating a recent study reporting high uL-FABP/Cr values in 18/34 (53%) cats with azotemic CKD.[26] As tubulointerstitial inflammation is a predominant feature of CKD in cats, upregulated uL-FABP was expected.[58] Moreover, uL-FABP/Cr levels correlate with the degree of tubulointerstitial damage and predicted CKD progression in humans.[21,26] Tubulointerstitial inflammation does not differ according to stage in cats with IRIS stage 2-4 CKD.[21,57] Hence, variations in uL-FABP/Cr may be because of different severities of tubular damage and may indicate a variable CKD progression rate in cats. The significant correlation between uL-FABP/Cr and sCr in this observation along with data in humans and CKD cats supports the hypothesis that uL-FABP concentrations increase as kidney function declines.[21,26] Still, given the limited number of CKD cats in the present study, it is difficult to determine a relationship between the severity of azotemia and uL-FABP/Cr values. A longitudinal study of uL-FABP in CKD cats including renal biopsy would allow the evaluation of its ability to monitor CKD progression.

Another possible cause for increased uL-FABP in CKD cats could be the effect from proteinuria since a significant correlation was observed between uL-FABP/Cr and UPC in the group of CKD cats and healthy cats. These results corroborate findings in humans with CKD.[20] The possible reason could be that massive proteinuria, which is uncommon in the cats of the present study and in the overall population of CKD cats,[59] causes tubulointerstitial damage by overloading proximal tubules with free fatty acids. This damages tubular cells and induces L-FABP excretion into urine.[20,60] An alternative explanation could be a lack of reabsorption mechanism of filtered L-FABP secondary to protein overload and the saturation of protein reuptake mechanism in proximal tubules, which is described for increased urinary N-acetyl-β-d-glucosaminidase excretion in CKD cats.[61]

TABLE 4 Spearman's correlations (ρ) between L-FABP and NGAL values and selected laboratory variables in hyperthyroid cats at before (T0), 1 month (T1), and 11 to 29 months after ^{131}I treatment (T2)

	sL-FABP		uL-FABP/Cr		sNGAL		uNGAL/Cr	
Variables	**ρ**	***P***	**ρ**	***P***	**ρ**	***P***	**ρ**	***P***
sCr								
T0	.18	.26	−.19	.17	−.14	.41	.22	.13
T1	.11	.49	.17	.24	−.10	.55	.06	.67
T2	.11	.62	.19	.35	−.35	.11	−.02	.92
UPC								
T0	−.06	.68	.66	<.001	.63	.16	.02	.87
T1	.20	.20	.31	.03	.02	.90	.30	.04
T2	.05	.83	.67	<.001	.28	.21	.78	<.001
GFR								
T0	−.63	.13	.58	.05	.42	.30	−.03	.27
T1	−.09	.80	.16	.62	.49	.18	−.36	.26
T2	.13	.68	.12	.72	−.04	.92	−.07	.83
sTT4								
T0	−.28	.75	.11	.47	.17	.75	−.16	.94
T1	.16	.32	−.10	.48	−.06	.87	.07	.63
T2	−.17	.44	−.09	.65	−.36	.10	.002	.99

Abbreviations: CKD, chronic kidney disease; GFR, glomerular filtration rate; L-FABP, liver-type fatty acid-binding protein; NGAL, neutrophil gelatinase-associated lipocalin; sCr, serum creatinine; sL-FABP, serum L-FABP; sNGAL, serum NGAL; sTT4, serum total thyroxine; uL-FABP/Cr, urinary L-FABP-to-creatinine ratio; uNGAL/Cr, urinary NGAL-to-creatinine ratio; UPC, urinary protein-to-creatinine ratio.

Corroborating a recent study, we found that most healthy cats have uL-FABP concentrations lesser than LOD except for 3 cats.[25,26] As noted for sL-FABP, early CKD cannot be completely excluded in these cats. In humans, uL-FABP is a highly sensitive marker of tubular injury, which makes uL-FABP a promising biomarker of early kidney disease.[21,22,62] Further studies on a large population of initially non-azotemic healthy cats should establish the ability of uL-FABP to detect early CKD in healthy cats.

Similar to a recent study in cats with various kidney conditions, sL-FABP concentration and uL-FABP/Cr were significantly correlated in the group of CKD cats and healthy cats, suggesting the variables are co-dependent in cats.[26] This contrasts to the finding in patients with CKD where sL-FABP and uL-FABP concentrations are not correlated.[55] Since sL-FABP is produced mainly by the liver, these findings suggest that liver disease that may increase sL-FABP concentration should be ruled out when interpreting uL-FABP/Cr as a renal biomarker in cats.[20] Further studies on the influence of nonrenal factors on L-FABP concentrations are warranted.

The sNGAL concentration did not statistically differ between CKD cats and healthy cats. Our data corroborate a recent study showing no difference in plasma NGAL (pNGAL) concentrations between CKD cats and healthy cats.[43] The median sNGAL concentrations in our study were much lower than the median pNGAL concentrations reported in the aforementioned study (31.33 ng/mL vs. 211.07 ng/mL in healthy cats)[43], probably because of the different ELISAs used in the 2 studies. More specifically, for pNGAL, an in-house ELISA was used based on an anticanine NGAL capture antibody, whereas we used a commercial ELISA with an antihuman NGAL antibody.[43] Although both of these capture antibodies are claimed to cross-react with feline NGAL, their affinities to feline NGAL are probably different. Indeed, homologies between human and feline NGAL and between canine and feline NGAL are 64% and 60%, respectively, using BLAST. Another explanation for the discordant results is that the high pNGAL concentration might be attributed to non-specific binding, since the accuracy of that ELISA was not provided. In addition, we observed different values between sNGAL and pNGAL in a preliminary study (Table S3).

Urinary NGAL/Cr did not significantly differ between CKD cats and healthy cats. In contrast, previous studies have shown that uNGAL/Cr is higher in CKD cats compared to healthy cats and even associated with IRIS stage.[43,63] The reason for these discrepant results is unclear. Corroborating a previous study, the present study demonstrated a poor correlation between sNGAL and uNGAL suggesting that, in cats, sNGAL does not influence uNGAL.[43]

ROC analysis demonstrated that L-FABP outperforms NGAL for the detection of azotemic CKD. More specifically, the ability of uL-FABP/Cr to detect azotemic CKD in CKD and healthy cats outperforms the ability of sL-FABP because of the greater sensitivity and specificity.

To our knowledge, the effect of naturally occurring hyperthyroidism on sL-FABP and uL-FABP levels has not been reported in any species. This study demonstrated that uL-FABP/Cr was increased in hyperthyroid cats and normalized after treatment. Although there was no significant difference in uL-FABP/Cr between hyperthyroid cats at

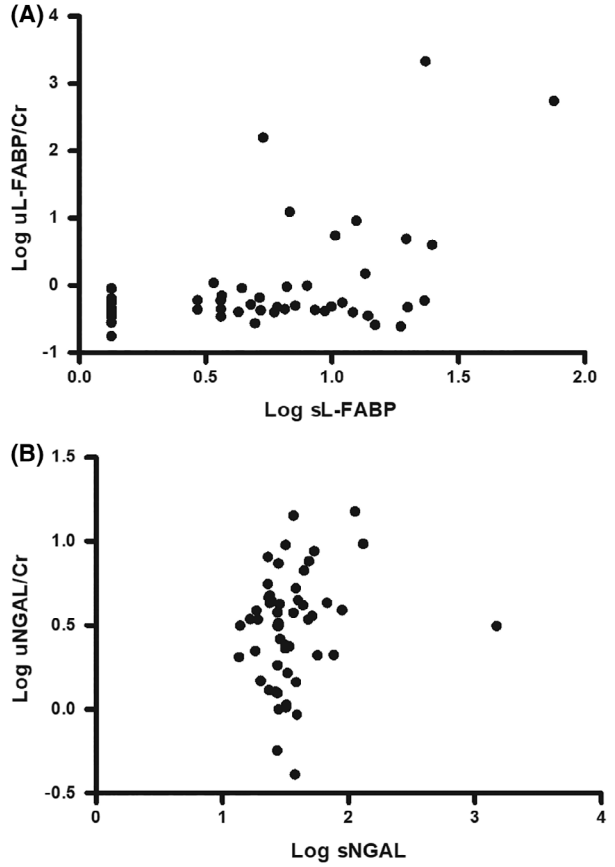

FIGURE 7 Spearman's correlation between serum and urinary biomarkers in cats. A, The correlation between log sL-FABP and log uL-FABP/Cr in the group of cats with CKD and healthy cats (n = 53). B, The correlation between Log sNGAL and Log uNGAL/Cr in the group of cats with CKD and healthy cats (n = 53)

T0 and T1, a significant difference was observed in uL-FABP concentration. Furthermore, the percentage of cats with uL-FABP concentration < LOD in hyperthyroid cats after treatment and in healthy cats were similar, suggesting that uL-FABP concentration in hyperthyroid cats generally normalizes after treatment. These findings are consistent with a number of studies in hyperthyroid cats evaluating other tubular biomarkers, which are all increased before treatment and normalize following treatment.[64-66]

Comparable to previous studies, our study showed uL-FABP's ability to detect CKD in cats. Therefore, it is possible that the increased uL-FABP concentration reflects masked CKD in cats with hyperthyroidism.[25,26] We found that uL-FABP concentration was greater than LOD in 35% of hyperthyroid cats before treatment, which was similar to the percentage of cats that has been reported to develop post-treatment azotemia (15%-40%).[21,55,67] Unfortunately, the very limited number of post-treatment azotemic cats in this study impedes the evaluation of uL-FABP to predict post-treatment azotemia. Another explanation could be that thyrotoxicosis might directly increase sL-FABP and filtered L-FABP concentrations, leading to an increase in uL-FABP concentration. One preclinical study in experimentally induced hyperthyroid rats shows an increased level of L-FABP in hepatocyte cytosol.[41] However, we observed poor correlations between sTT4 and both sL-FABP and uL-FABP/Cr and between sL-FABP and uL-FABP/Cr in hyperthyroid cats before and after treatment. Also, sL-FABP concentration in hyperthyroid cats did not differ from healthy cats and remained unchanged after treatment. Therefore, the direct effect of hyperthyroidism to increased uL-FABP in cats remains unclear. Alternatively, increased uL-FABP excretion may reflect a lack of reuptake mechanism of filtered L-FABP in urine because of proteinuria.[61] Supporting this hypothesis, our study found a moderate and significant association between UPC and uL-FABP in hyperthyroid cats before and after treatment.

Neither sNGAL concentrations nor uNGAL/Cr values differed significantly between hyperthyroid cats and healthy cats in the cross-sectional study. These results show that hyperthyroidism may not affect sNGAL and uNGAL concentration in cats. However, in the longitudinal study, sNGAL concentration in hyperthyroid cats before treatment was significantly decreased upon euthyroidism. One report in humans also observes no significant difference in pNGAL levels between hyperthyroid and euthyroid patients.[68] With a very limited data regarding the sNGAL expression in cats and other species with hyperthyroidism, the reason of these conflicting results remains unclear. Currently, there are no data regarding the relationship between thyroid dysfunction and uNGAL expression.

A limitation of our study is the limited number of CKD. Also, only 2 hyperthyroid cats developed post-treatment azotemia we cannot conclude on the prediction of post-treatment azotemia. Post-treatment azotemia in hyperthyroid cats treated with fixed dose protocols of ^{131}I is present in 15% to 46% of all cats.[48,69] The lower number of post-treatment azotemic cats (8%) in this study is probably because of the adjusted dose protocol of ^{131}I used at our university for many years, which is based on the severity of hyperthyroidism and size of the thyroid glands in each cat. In contrast to the fixed dose method, the adapted dose protocol may give more suitable dose of ^{131}I for hyperthyroid cats and may result in lower number of cats with iatrogenic hypothyroidism and post-treatment azotemia.[70] Thirdly, the timing of the assessment at T2 (11-29 months) was variable between cats. This may have affected the variability of the measured variables at this time point. The values of L-FABP and NGAL may increase at the time of tubular injury and gradually decrease over time so there is a possibility that we missed the time frame when these biomarkers reached their peak levels.[25,37] Another potential limitation was that a long-term frozen storage might have affected the measured concentrations of both L-FABP and NGAL. However, in humans, uL-FABP concentrations do not significantly change after storage at −70°C for 18 months.[71] Again, in human samples, urinary NGAL is found to be stable at −80°C when stored for up to 5 years.[72] Lastly, the antibodies in the feline L-FABP and NGAL ELISA kits used in this study were antihuman, consequently the obtained concentrations are relative to humans instead of absolute concentrations in cats. Hence, comparisons of L-FABP and NGAL concentrations and ratio to Cr within species are acceptable, but not between humans and cats.

5 | CONCLUSION

Only urinary L-FABP/Cr distinguished CKD cats from healthy cats, suggesting that uL-FABP might be a promising biomarker of early renal impairment in cats. The increased uL-FABP concentration in hyperthyroid cats probably reflects renal insult in hyperthyroidism and resolved when euthyroidism was obtained. Nevertheless, the predictive value of uL-FABP for prediction of post-treatment azotemia in hyperthyroid cats remains uncertain. In contrast to uL-FABP/Cr, NGAL in both serum and urine is not a useful biomarker of renal dysfunction in cats.

ACKNOWLEDGMENT

Funding provided by IDEXX Laboratories Thirawut Kongtasai is supported by a grant from Mahidol University, Thailand, for studying the doctoral program at Ghent University. Results were partially presented at ECVIM-CA online congress, 2020.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

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 2015/68 and EC 2017/72).

HUMAN ETHICS APPROVAL DECLARATION

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

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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: Kongtasai T, Meyer E, Paepe D, et al. Liver-type fatty acid-binding protein and neutrophil gelatinase-associated lipocalin in cats with chronic kidney disease and hyperthyroidism. *J Vet Intern Med*. 2021;1–13. https://doi.org/10.1111/jvim.16074