Research Paper

Plasma vitamin K-dependent protein C as early diagnostic marker of feline chronic kidney disease

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Abstract Chronic kidney disease (CKD) is a common disorder and cause of death in cats. In the classification proposed by the International Renal Interest Society (IRIS), stage 1 and 2 CKD are difficult to diagnose accurately using markers, in comparison with normal control cats. We recently described a simple and highly reproducible tandem mass tag (TMT) labelling method for identifying potential disease-marker candidates among low-abundance plasma proteins. In the current study, plasma samples were obtained from 90 normal control cats and 50 cats with CKD (stage 1). To identify new plasma biomarkers for CKD, six plasma samples (three from normal control cats and three from CKD stage 1 cats) were extracted with SSA lectin magnetic beads, differentially labelled with TMTs, digested with trypsin and subjected to analysis using LC-MS/MS. Sialylated Vitamin K-dependent protein C was identified as a protein with lower levels in CKD stage 1 cats compared to normal control cats. An ELISA of plasma sialylated vitamin K-dependent protein C showed within-run (4.3−4.9%) and between-day (4.3−5.2%) reproducibility. Plasma sialylated vitamin K-dependent protein C levels measured with this assay were significantly greater in normal control cats than in CKD stage 1 cats (1.40±0.13 vs. 1.07±0.11 AU/mL, $p < 0.001$). These results indicate that sialylated vitamin K-dependent protein C may be useful as a complementary marker with plasma creatinine, BUN and SDMA for detection of CKD stage 1 in cats.

Key words: chronic kidney disease, feline, plasma, vitamin K-dependent protein C

Introduction

Feline renal diseases are increasingly common in veterinary practice[1)]. It is important to diagnose and identify the pathological basis of renal dysfunction accurately at an early stage, since early diagnosis and treatment can delay progression of renal dysfunction, which leads to prolongation of survival and improvement of QOL[2)]. However, there are only a few reports on this area in clinical veterinary medicine[3)].

In daily clinical practice, plasma creatinine (p-Cre) and serum symmetric dimethylarginine (SDMA) are used as markers based on the stage classification criteria of the International Renal Interest Society (IRIS)[4)]. However, many of these markers have low specificity for renal function and there is a blind area without abnormal values, despite development of mild renal dysfunction. Therefore, it is especially important to evaluate the transitional period from a normal status to Stage 1 in the IRIS classification for early diagnosis of renal function. However, it is difficult to identify a mild decrease of renal function in the early phase using the available markers in clinical practice. In stage 1 there may not be any reduction in GFR or surrogate

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markers of GFR, despite ongoing active disease in the kidney.

We recently described a simple and highly reproducible tandem mass tag (TMT) labelling method for identifying potential disease-marker candidates among low-abundance plasma proteins[8]. In this study, we used the lectin and tandem mass tag (TMT) method to generate comparative protein profiles of plasma samples from normal control cats and CKD stage 1 cats. Plasma levels of candidate proteins were compared to evaluate their potential to discriminate between the normal control cats and CKD cats. Novel plasma biomarkers were identified that may be superior to classical markers for early and specific detection of CKD stage 1 in cats.

Materials and Methods

Animals

Plasma samples from 50 cats with stage 1 CKD and 10 cats with stage 2 CKD brought to Maeda Veterinary Hospital between January 6, 2017 and February 20, 2018 (American Shorthair: 5, Chinchilla: 2, Mix: 53; 29 males and 31 females; age 2−13 years) were used in the study. Plasma samples from 90 cats aged 2−13 years (Scottish Fold: 4, American Shorthair: 2, Mix: 84; 43 males and 47 females) were used as normal control cats. Cats clinically diagnosed as normal based on serum biochemistry and ultrasonography tests were defined as normal cats, and cats showing clinical signs of CKD in blood chemistry were defined as those with stage 1 CKD. Criteria for normal urinalysis were urine specific gravity (USG) ＞1.030, urinary protein to creatinine ratio (UPC) ≤0.2, and a negative bacteriologic urine culture[5]. Diagnosis of CKD was made prior to inclusion in the study, based on clinical and laboratory findings. After stabilization, cats were classified[5] into 2 groups with p-Cre of ＜1.6 and 1.6−2.8 mg/dL in stages 1 and 2, based on the IRIS guidelines. Diagnosis of stage 1 CKD was made if p-Cre was ＜1.6 mg/dL, urine concentrating ability had been lost, other disorders related to decreased urine concentrating ability were excluded, and ultrasonographic changes were consistent with CKD (e.g., small kidney size and poor or absent renal corticomedullary differentiation)[6]. Samples were centrifuged at 1,710×*g* for 10 min (Model 6200; Kubota, Tokyo, Japan) and then stored at −80°C for further use. The ethics committee for Maeda Veterinary Hospital approved the protocol (2016-02). All owners gave signed informed consent to participation of their animal in the study.

Magnetic bead sample preparation using the Glycoprotein Isolation Kit

Paramagnetic non-porous particles coupled with a sambucus sieboldiana lectin ligand (MB-SSA; Bruker Daltonics, Bremen, Germany) were used to process the plasma samples. Binding, washing and elution solutions were prepared according to the manufacturer's instructions. MB-SSA binding solution (10 μL) and plasma (5 μL) were transferred to a 0.2− mL thin-walled tube. A homogenous magnetic-particle solution (10 μL) was added, mixed, and incubated for 5 min. The tubes were placed in an 8×12-well magnetic-bead separator (MBS; Bruker Daltonics) for 30 sec for magnetic fixation of the MB-SSA particles. The supernatant was aspirated and the tubes were removed from the MBS device. Washing solution (100 μL) was added and carefully mixed with the magnetic beads. The tube was placed back in the MBS device and moved back and forth sequentially between adjacent wells on each side of the magnetic bar of the device. After fixation of the magnetic beads for 30 s, the supernatant was aspirated. This washing procedure was repeated three times. After the final washing step, the bound molecules were eluted by incubation with 10 μL MB-SSA elution solution for 1 min, before the eluate was collected using the MBS device.

Tandem mass tag (TMT) labelling

Reduction and alkylation were performed as described previously[7]. A TMT sixplex Isobaric Label Reagent Set (Thermo Scientific, Rockford, IL, USA) was used according to the manufacturer's instructions. Tubes containing the different isobaric chemical tags (0.8 mg each) were added to 41 μL of anhydrous acetonitrile and dissolved for 5 min with occasional vortexing at room temperature. TMT solution (20 μL) was added to each tube and allowed to react at room temperature for 60 min. A volume of 4 μL of 5% hydroxylamine was added to each sample and the mixture was incubated for 15 min to quench the reaction[7]. Samples were finally pooled and lyophilized. Analyses were performed for samples of 20 μL of glycoprotein-isolated plasma from normal control cats (n=3) (TMT6-126, 127 and 128) and stage 1 CKD cats (n=3) (TMT6-129, 130 and 131). Plasma samples were randomly drawn.

Protein identification and quantification by LC-MS/MS analysis

Trypsin-digested peptides were injected into a trap col-

umn (C18, 5μm, 0.3×5mm) (IonPac; Thermo Scientific, Rockford, IL, USA) and an analytical column (C18, 3 μm, 0.075×120mm) (C18 packed emitter column；Nikkyo Technos, Tokyo, Japan) attached to an HPLC system (Ultimate 3000; Thermo Scientific). The flow rate of the mobile phase was 300nL/min. The solvent composition of the mobile phase was programmed to change in 120min cycles, with varying mixing ratios of solvent A (2% acetonitrile and 0.1% formic acid) to solvent B (90% acetonitrile and 0.1% formic acid), as described previously[7]. Purified peptides from HPLC were moved to a hybrid ion-trap Fourier transform mass spectrometer (LTQ-Orbitrap XL; Thermo Scientific) and analyzed using previously established parameters[8]. A database search engine (Proteome Discoverer; Thermo Scientific) was used to identify and quantify proteins from the mass, tandem mass and reporter ion spectra of peptides. Peptide mass data were matched by searching the UniProt database (https://www.uniprot.org/proteomes/?query=*) using the search parameters: peptide mass tolerance=2ppm and fragment tolerance=0.6Da, with the enzyme parameter set to trypsin, allowing up to one missed cleavage variable modification, methionine oxidation, and cysteine alkylation. The minimum criteria for protein identification were filtered with Xcorr vs. charge state and a false discovery rate (FDR) ＜1%.

ELISA

Immunization and establishment of hybridoma cell lines

Synthetic peptides of 20 amino acids (vitamin K-dependent protein C N peptide; FUJIFILM, Osaka, Japan) were used for immunization of BALB/c mice. Hybridoma cell line vitamin K-dependent protein C N-01[9] was established and antibody isotypes were determined using a Mouse Monoclonal Antibody Isotyping Test Kit (AbD Serotec, Kidlington, United Kingdom). To obtain pure monoclonal antibodies on a large scale, BALB/c mice were initially stimulated with 1.0mL pristine (Sigma-Aldrich, St. Louis, MO, USA) and then inoculated 2 weeks later. Monoclonal antibodies were purified as described elsewhere[9].

Immobilization of antibodies on a polystyrene microtiter plate

The anti-vitamin K-dependent protein C N peptide antibody dissolved in PBS buffer was dispensed into a 96-well polystyrene microtiter plate (Thermo Scientific, Rockford, IL, USA) at 0.5mg/well and incubated for 1 day at 4˚C. The plate was washed three times with PBS containing 0.05% Tween-20 (PBST). The microtiter plate was coated with 20% NOF102 containing 10% sucrose for 1 day at 4˚C.

ELISA conditions

After washing the microtiter plate with PBST, 100-μL aliquots of 10-times diluted plasma samples were added in duplicate to wells. The plates were incubated at room temperature for 1h and then washed three times. HRP-conjugated SSA lectin (Vector Laboratories, Burlingame, CA, USA) in PBST (100μL) was added to each well and the plate was incubated at room temperature for 30min. The plate was washed three times and then 100μL of TMB solution (FUJIFILM, Osaka, Japan) was added. After incubation at room temperature for 10min, 100μL of stop solution was added and the absorbance at 450nm was measured.

Other procedures

Creatinine was measured enzymatically with creatinine deiminase using a Fuji DRI-CHEM Slide CRE-PIII kit (FUJIFILM, Osaka, Japan). Blood urea nitrogen (BUN) was measured using a N-Assay BUN-L Nittobo D-Type kit (Nittobo Medical, Tokyo, Japan). Plasma SDMA was measured using a Cat SDMA ELISA Kit (MyBioSource, San Diego, CA, USA). Serum levels of vitamin K-dependent protein C was determined by enzyme-linked immunosorbent assays according to the manufacturer's instructions (ELISA Kits for vitamin K-dependent protein C, LifeSpan Biosciences. Seattle, WA, USA).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 19 (SPSS, Chicago, IL, USA). Numerical data are presented as the mean±standard deviation (SD). Differences in non-parametric data between two groups were analyzed by Mann-Whitney U-test, three groups were analyzed by Steel-Dwas test, with $P<0.05$ considered significant. Receiver operating characteristic (ROC) curves were constructed to assess the sensitivity, specificity, and areas under the ROC curves (AUCs).

Results

Identification of candidate proteins as markers of CKD stage 1

Clinical characteristics and biochemical variables of the

normal control cats and CKD groups are shown in Table 1. To identify new plasma biomarkers for CKD, six plasma samples (three normal control cat and three CKD stage 1) were alkylated with iodoacetamide, digested with trypsin, differentially labelled with TMTs, pooled and subjected to analysis using LC-MS/MS (Fig. 1). The three normal control cat samples were labelled with TMTs with reporter ions at *m/z*=126, 127 and 128, and the three CKD stage 1 samples were labelled with TMTs with reporter-ions at *m/z*= 129, 130 and 131. After comparing the profiles of proteins by LC-MS/MS using an LTQ Orbitrap XL mass spectrometer, 18 sialylated glycoproteins with unique peptide sequences were found with higher levels in normal control cats compared to CKD stage 1 cats (Table 2). Among these proteins, hemoglobin subunit alpha and vitamin K-dependent protein C had plasma levels elevated more than two-fold in all three normal control cats compared to the CKD stage 1 cats. Vitamin K-dependent protein C was selected for further analysis.

Establishment of an ELISA for sialylated vitamin K-dependent protein C

Range, dilution analysis and detection limit

A standard curve was drawn based on the colorimetric intensity of diluted recombinant cat vitamin K-dependent protein C (Cusabio Technology) to establish the relationship of intensity with the sialylated vitamin K-dependent protein C concentration (Fig. 2). The assay had a working range of 0−10AU/mL and gave linear results from 0 to 10AU/mL ($y=0.021\times-0.0018$, $r^2=0.9993$, $p<0.0001$). The detection limit was estimated by assaying the zero concentration eight times, and was defined as the sialylated vitamin K-dependent protein C "zero" concentration+3SD. The limit was found to be 0.18AU/mL.

Within-run and between-run reproducibility

The precision of the assay was determined using two sialylated vitamin K-dependent protein C concentrations of 0.5 and 2.0AU/mL. Within-assay CVs were determined with eight replicates of each sample. Between-assay CVs

Table 1. Clinical characteristics and biochemical variables of normal control cats and CKD cats

Variables	Normal control cats	CKD group		p-Value
		Stage 1	Stage 2	
Age (Mean±s.d.)	6.8±3.6	6.7±3.3	6.8±3.6	0.825
Sex (male, female)	90 (43, 47)	50 (24, 26)	10 (5, 5)	0.793
Blood urea nitrogen (mg/dL) (Mean±s.d.)	24.1±5.7	25.6±6.8	26.8±5.9	0.714
p-Creatinine (mg/dL) (Mean±s.d.)	0.96±0.23	1.20±0.23	1.99±0.24	0.015
Symmetric dimethylarginine (mg/dL) (Mean±s.d.)	5.9±0.8	6.4±1.0	10.9±1.6	0.009
Urine specific gravity (Mean±s.d.)	1.053±0.006	1.051±0.005	1.045±0.016	0.618
Urinary protein to creatinine ratio (Mean±s.d.)	0.13±0.04	0.15±0.05	0.17±0.06	0.024

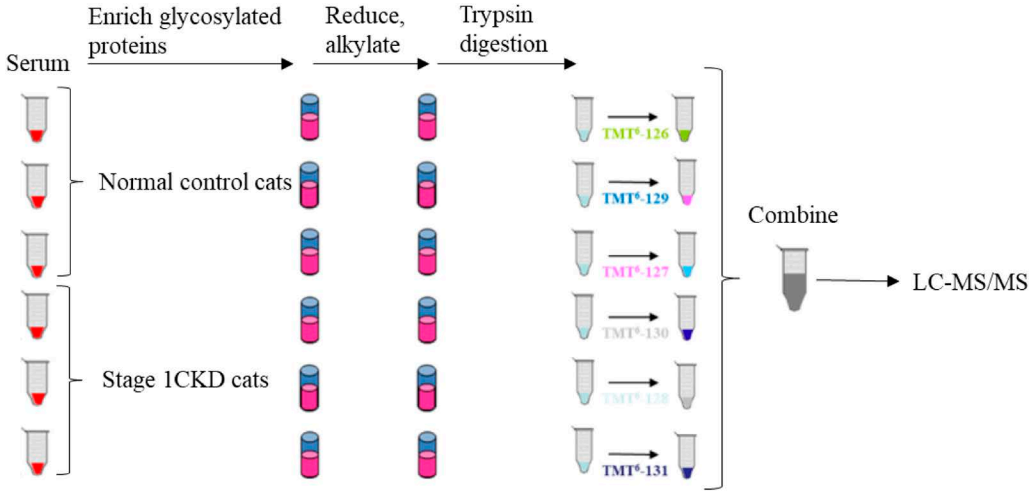

Fig. 1. Identification of candidate proteins using TMT labelling and LC-MS/MS analyses.

Table 2. Plasma sialylated glycoproteins detected at higher levels in normal control cats compared to CKD stage 1 cats in proteome analysis

Database Accession No.	ID	Coverage (%)	Number of matching peptides	TMT							
				Normal control cats				Stage 1 CKD cats			
				126	127	128	Average	129	130	131	Average
M3W3E7	Alpha-2-macroglobulin	3.46	6	0.268	0.233	0.226	0.242	0.206	0.134	0.152	0.164
M3WPG6	Apolipoprotein A-I	12.41	4	0.298	0.240	0.167	0.235	0.236	0.108	0.139	0.161
M3WG78	ATP synthase subunit beta	8.00	3	0.212	0.158	0.133	0.168	0.277	0.106	0.113	0.166
M3W7M4	Attractin	4.69	6	0.260	0.134	0.307	0.234	0.180	0.164	0.133	0.159
M3WB05	Bifunctional purine biosynthesis protein ATIC	2.86	2	0.357	0.164	0.154	0.225	0.282	0.122	0.121	0.175
M3VZY7	Ceruloplasmin	5.63	4	0.230	0.142	0.268	0.213	0.230	0.136	0.142	0.169
M3WKP2	Clusterin	16.63	13	0.391	0.132	0.263	0.262	0.140	0.157	0.140	0.146
M3VU42	Complement factor I	2.31	2	0.394	0.162	0.227	0.261	0.106	0.263	0.219	0.196
P14450	Fibrinogen alpha chain (Fragment)	100	1	0.191	0.191	0.245	0.209	0.198	0.160	0.175	0.178
P14469	Fibrinogen beta chain	5.43	3	0.217	0.237	0.251	0.235	0.270	0.214	0.156	0.213
M3WB06	Fibronectin	4.24	8	0.194	0.155	0.270	0.207	0.297	0.181	0.131	0.203
P07405	Hemoglobin subunit alpha	18.44	2	0.480	0.336	0.198	0.338	0.167	0.130	0.133	0.143
M3WEG4	Phosphatidylinositol-glycan-specific phospholipase D	3.93	3	0.487	0.114	0.220	0.274	0.122	0.130	0.224	0.159
P49064	Serum albumin	31.58	19	0.251	0.177	0.130	0.186	0.278	0.131	0.128	0.179
M3WEV9	Transthyretin	8.84	2	0.169	0.121	0.188	0.159	0.118	0.189	0.116	0.141
M3WFT7	UDP-glucose 4-epimerase	2.66	2	0.152	0.208	0.217	0.192	0.251	0.174	0.099	0.174
Q28412	Vitamin K-dependent protein C	14.21	3	0.280	0.291	0.289	0.287	0.132	0.140	0.137	0.136
M3W9I5	Vitronectin	5.47	2	0.337	0.343	0.388	0.356	0.236	0.197	0.186	0.206

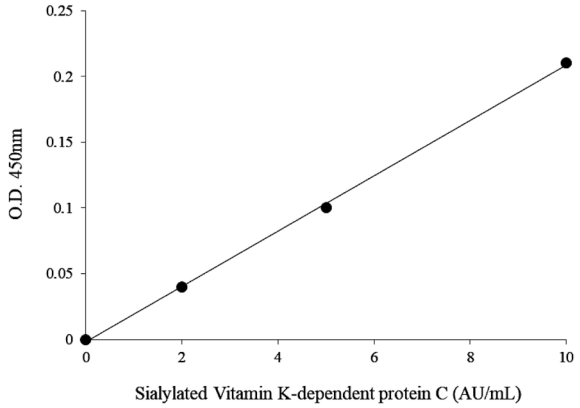

Fig. 2. Standard curves for plasma sialylated vitamin K-dependent protein C in the ELISA.
Colorimetric intensity and plasma vitamin K-dependent protein C concentration were related in the range of 0−10 AU/mL. Four concentrations of plasma sialylated vitamin K-dependent protein C were determined by ELISA.

were determined based on assays performed on 5 different days (two replicates of each sample per day). The within-run CV was 4.3−4.9% and the between-run CV was 4.3−5.2%.

Interference

Interference was assessed in samples containing 1.0 AU/mL sialylated vitamin K-dependent protein C. Potential interference materials were added to urine at various concentrations. There was no substantial interference from hemoglobin (up to 5000 mg/L), free bilirubin (up to 207 mg/L), ditaurobilirubin (up to 204 mg/L), chyle (up to 1400 formazine turbidity units, equal to 1176 mg/L triglyceride), ascorbic acid (up to 500 mg/L), and rheumatoid factor (up to 500 U/L).

Recovery test

To evaluate recovery in the ELISA, 0.5 and 2.0 AU/mL of recombinant Cat vitamin K-dependent protein C were added to pooled plasma (1.6 AU/mL). The percentage recovery ranged from 94.3 to 102.7%.

Sialylated vitamin K-dependent protein C levels are decreased in plasma of cats with CKD

Plasma vitamin K-dependent protein C levels and sialylated vitamin K-dependent protein C levels were mea-

sured in stage 1 CKD cats (n=50), stage 2 CKD cats (n=10) and normal control cats (n=90). Plasma vitamin K-dependent protein C values for normal control cats, stage 1 CKD cats and stage 2 CKD cats were 3.36±0.32 μg/mL, 3.46±0.31 μg/mL and 3.53±0.30 μg/mL, respectively (Fig. 3). Plasma sialylated vitamin K-dependent protein C was significantly higher in normal control cats than in stage 1 CKD cats (1.40±0.13 vs. 1.07±0.11 AU/mL, $p<0.001$), and in stage 1 CKD cats compared to stage 2 CKD cats (1.07±0.11 vs. 0.82±0.14 AU/mL, $p<0.001$) (Fig. 4). ROC curves were constructed to evaluate the cut-offs for plasma sialylated vitamin K-dependent protein C, plasma creatinine, BUN, and SDMA for distinguishing stage 1 CKD cats from normal control cats. The AUCs were 0.989 for plasma sialylated vitamin K-dependent protein C, 0.698 for plasma creatinine, 0.554 for BUN, and 0.568 for SDMA. These results suggest that plasma sialylated vitamin K-dependent protein C may be a better diagnostic stage 1 CKD biomarker than plasma creatinine, BUN and SDMA (Fig. 5). The ROC curve for plasma sialylated vitamin K-dependent protein C showed an optimal diagnostic cut-off of 1.22 AU/mL and an AUC of 0.989, with sensitivity of 97.5% and specificity of 97.5%.

Discussion

In this study, the lectin and TMT method was used to detect 18 proteins in plasma from CKD stage 1 cats and normal control cats. Sialylated vitamin K-dependent protein C was identified as the protein with the largest difference in levels between the two groups. The vitamin K-dependent protein C is synthesized in the liver in a single-chain form, and through several proteolytic steps is secreted into blood primarily as a two-chain form, containing a light chain and a heavy chain linked via a single disulfide bond[10]. Sialic acids are derivatives of the negatively charged acidic sugar neuraminic acid. Over 40 naturally occurring members of the sialic acid family have been discovered to date[11]; N-acetylneuraminic acid, N-glycolylneuraminic acid, and deaminoneuraminic acid are representative members of this family. Sialic acids usually form the terminal ends of the carbohydrate groups of glycoconjugates. All sialyltransferases characterized in animals to date, ranging from insects

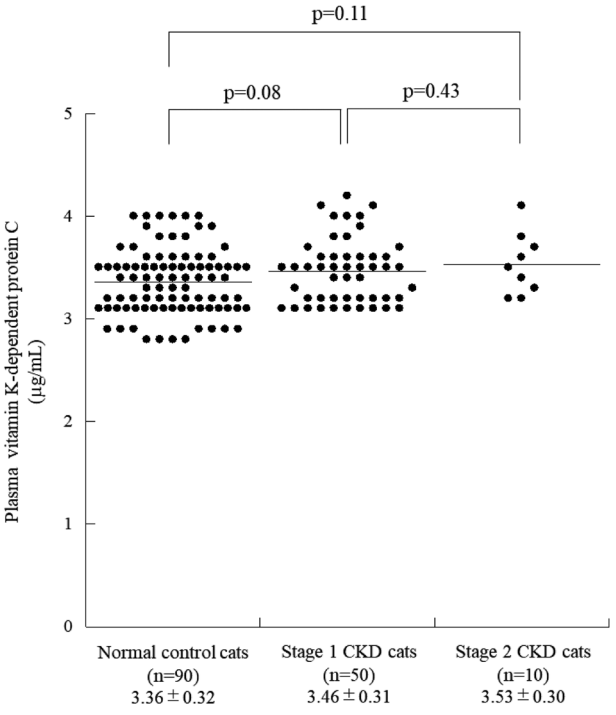

Fig. 3. Plasma vitamin K-dependent protein C levels in normal control cats and stage 1 and stage 2 CKD cats.
Plasma vitamin K-dependent protein C values for normal control cats, stage 1 CKD cats and stage 2 CKD cats were 3.36±0.32 μg/mL, 3.46±0.31 μg/mL and 3.53±0.30 μg/mL, respectively.

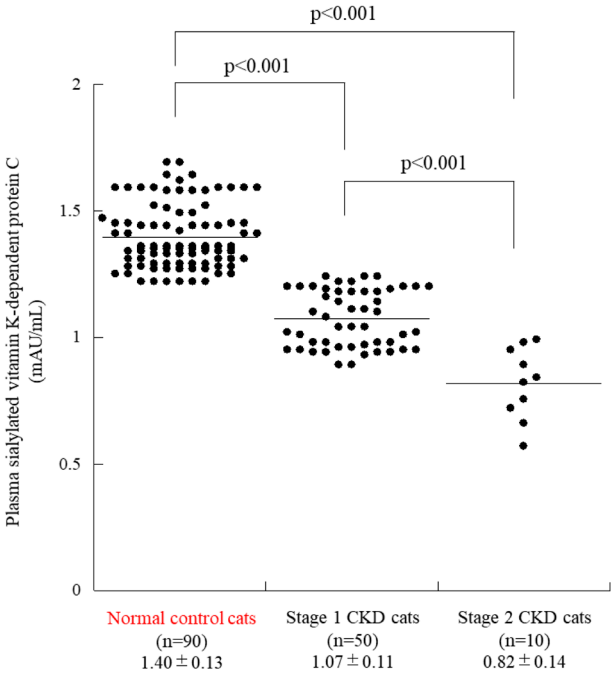

Fig. 4. Plasma sialylated vitamin K-dependent protein C levels in normal control cats and stage 1 and stage 2 CKD cats.
Plasma sialylated vitamin K-dependent protein C was significantly higher in normal control cats than in stage 1 CKD cats (1.40±0.13 vs. 1.07±0.11 AU/mL, $p<0.001$; Mann−Whitney *U*-test) and in stage 1 CKD cats compared to stage 2 CKD cats (1.07±0.11 vs. 0.82±0.14 AU/mL, $p<0.001$).

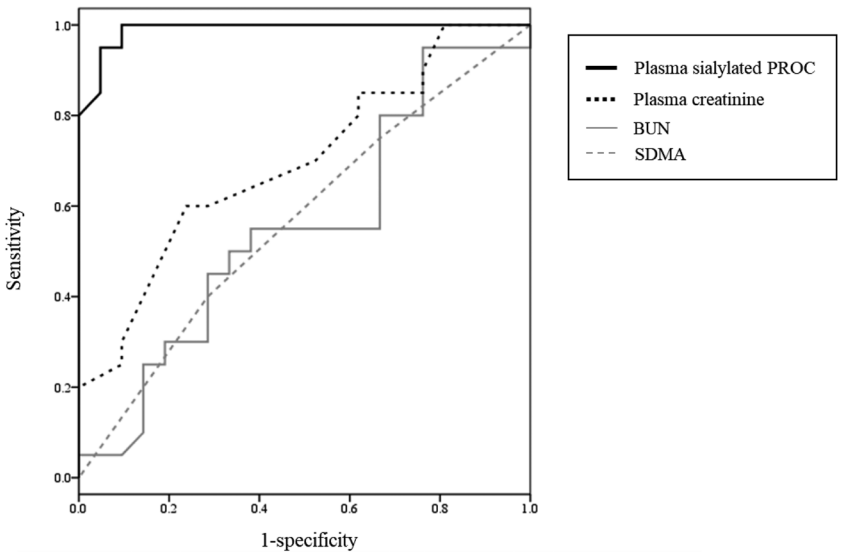

Fig. 5. Plasma sialylated vitamin K-dependent protein C is more effective than plasma creatinine, BUN and SDMA for early detection of stage 1 CKD.

ROC analyses were performed for serum levels of plasma sialylated vitamin K-dependent protein C, plasma creatinine, BUN and SDMA in stage 1 CKD and normal cats. The AUCs were 0.989 for plasma sialylated vitamin K-dependent protein C, 0.698 for plasma creatinine, 0.554 for BUN, and 0.568 for SDMA.

to mammals, feature a type II transmembrane topology and usually localize to the Golgi apparatus. The enzyme is grouped into 4 families according to the type of carbohydrate linkage they synthesize: β-galactosideα2,3-sialyltransferases (ST3Gal-I-VI), β-galactosideα2,6-sialyltransferases (ST6Gal-I and -II), GalNAcα2,6-sialyltransferases (ST6GalNAc-I−VI), and α2,8-sialyltransferases (ST8Sia-I−VI). Sialic acid was added by these enzymes[12]. During coagulation, plasma vitamin K-dependent protein C is activated by a thrombin/thrombomodulin complex[11], and in the presence of protein S, activated protein C (APC) functions as an anticoagulant by proteolytic degradation of factors Va and VIIIa[12], the only protein substrates hitherto identified for vitamin K-dependent protein C. Several animal models, including canine, bovine and feline vitamin K-dependent protein C, have been used to examine the physiological mechanisms of vitamin K-dependent protein C, and species-specific effects have been found for the anticoagulant and fibrinolytic functions of APC[13,14]. Thus, in squirrel monkeys, human APC has an anticoagulant effect, but no profibrinolytic activity[15], whereas human APC shows both anticoagulant and fibrinolytic responses in cats[16].

A decrease in vitamin K-dependent protein C has been reported in renal disease in humans, but not in cats. Patients with CKD commonly have blood coagulation disorders, and several tests can be used to measure procoagulant biomarkers in CKD[17]. Compared to normal control cats, patients with CKD have significantly higher FVII, FVIII, fibrinogen and D-dimer levels[18] and a significantly lower level of protein C. However, it has not been reported in the sialylated vitamin K-dependent protein C. Similar results were obtained in this study, with plasma sialylated vitamin K-dependent protein C found to be significantly higher in normal control cats (1.40±0.13 AU/mL) than in stage 1 (1.07±0.11 AU/mL) and stage 2 (0.82±0.14 AU/mL) CKD cats.

In this study, we showed that plasma sialylated vitamin K-dependent protein C is a potential biomarker for CKD. Various attempts have been made to identify new plasma biomarkers for CKD, and some molecular targets have been suggested as diagnostic or prognostic markers[5]. The AUC for plasma sialylated vitamin K-dependent protein C was 0.989, with sensitivity of 97.5% and specificity of 97.5%. These results suggest that plasma sialylated vitamin K-dependent protein C may be complementary to plasma creatinine, BUN and SDMA as a marker for detection of stage 1 CKD in cats. We note that the study is limited by the number of samples, and evaluation of more samples from multiple facilities is required. Longitudinal follow-up of cats in stage 1 CKD is also important to identify those with true CKD, which is progressive, and those recovering from AKI when samples were taken.

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Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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