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Biological variation of major gut-derived uremic toxins in the serum of healthy adult cats

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Abstract

Background: Biological variation of serum indoxyl sulfate (IS), p-cresol sulfate (pCS), and trimethylamine-n-oxide (TMAO) concentrations in cats is unknown.

Objectives: To determine short- and medium-term biological variation, index of individuality (II), and reference change values for serum IS, pCS, and TMAO concentrations in healthy adult cats. To determine the effect of feeding on serum concentrations.

Animals: Twelve healthy adult cats.

Methods: Prospective, cohort study. Seven serum samples over a 12-hour period (short-term) and 5 serum samples over a 19-day period (medium-term) were collected. Serum concentrations of total IS, pCS, and TMAO were measured every 2 hours in a 12-hour period (hours 0-12) after a meal in 9 cats and compared to concentrations in a nonfed state.

Results: For IS, the II was high using short-term (1.96) and low using medium-term (0.65) biological variation estimates. Individuality was intermediate for pCS (short-term, 0.98; medium-term, 1.17) and TMAO (short-term, 1.47; medium-term, 0.83). Serum IS, pCS, and TMAO concentrations were significantly lower in a fed state compared to a nonfed state at hours 4, 6, 8, and 12; at hours 4 and 6; and at hours 2, 4, 6, 8, 10, 12, respectively.

Conclusion and Clinical Importance: Population-based reference intervals with reference to the subject-based interval can be used to monitor serum pCS and TMAO concentrations. For IS, a subject-based and a population-based reference interval is best for short-term and medium-term monitoring, respectively. To compare serial measurements, it would be prudent to collect samples at the same time of day and consistently in either a fed or nonfed state.

KEYWORDS
index of individuality, indoxyl sulfate, p-cresol sulfate, reference change value, trimethylamine-n-oxide

Abbreviations: CKD, chronic kidney disease; CV_A, analytical variation; CV_G, group variation; CV_I, within-individual variation; II, index of individuality; IS, indoxyl sulfate; LS-MS/MS, liquid chromatography tandem mass spectrometry; pCS, p-cresol sulfate; RCV, reference change value; TMAO, trimethylamine-n-oxide.

1 | INTRODUCTION

In people with chronic kidney disease (CKD), the accumulation of the major gut-derived uremic toxins indoxyl sulfate (IS), p-cresol sulfate (pCS), and trimethylamine-n-oxide (TMAO) in systemic circulation is, in part, a consequence of functional dysbiosis.[1,2] Assessment of the concentrations of these compounds, in particular IS and pCS, in people, dogs, and cats with CKD provides information on disease stage and risk of disease progression.[3-5] Additionally, these compounds have been used as biomarkers of therapeutic dietary intervention in people.[6-8] Liquid chromatography alone or in combination with mass spectrometry are common methods to measure IS, pCS, and TMAO in plasma or serum.[9,10]

Understanding biological variation and knowing how to interpret data is of paramount importance for both research purposes and to assess clinical utility of measuring IS, pCS, and TMAO in cats. Analytical measurements all have random and nonrandom variability from multiple sources. In addition to expected changes in the physiological status of the individual and analytical imprecision, biological variation is a source of variability and describes the physiological random fluctuation around a homeostatic set point. Assessment of biological variation for an analyte is necessary in order to interpret clinical test results, including whether a population-based reference interval is appropriate for the analyte.[11,12]

Estimates of biological variation include within-individual variation (CV_I), after accounting for analytical variation (CV_A), and between-individual (or group) variation (CV_G). Biological variation studies are most commonly performed on healthy individuals because often estimates are constant across age, geography, disease state, and methodology.[13] An understanding of biological variation allows practitioners to determine if a change in a measured analyte is clinically relevant (ie, reference change value [RCV]), even if the value is within the population-based reference interval. To determine the applicability of population-based reference intervals, the index of individuality (II) must be determined for the analyte. The II describes the relationship between CV_I and CV_G and takes into account CV_A.[11,12]

Although a reference interval and biological variation estimates have been established in healthy people for serum total IS, pCS, and TMAO,[14,15] evaluation of biological variation for these compounds has not been explored in veterinary medicine. In addition, it is unknown whether recent feeding affects serum concentrations of IS, pCS, and TMAO in cats. Therefore, the primary aim of this study was to determine biological variation estimates, II, and RCVs for serum total IS, pCS, and TMAO concentrations in healthy adult cats. The secondary aim was to measure the difference in serum concentrations in samples from cats that were fed and after feed had been withheld to determine optimal testing conditions to use in the field. The hypothesis was that subject-based reference intervals should be used for monitoring of changes in serial results and that serum concentrations will gradually increase after feeding.

2 | MATERIALS AND METHODS

2.1 | Study design and animals

The prospective cohort study was performed in 3 parts (Figure 1). In part 1, short-term biological variation estimates of serum concentrations of total IS, pCS, and TMAO were determined. Serum samples were collected every 2 hours over a 12-hour period after feed had been withheld for 12 hours. In part 2, serum samples were collected every 2 hours over a 12-hour period after a meal. In part 3, medium-term biological variation estimates of the 3 analytes were determined after analyzing results from samples collected after feed had been withheld 5 times over a 19-day period.

Based on the recommendation of previously published guidelines,[12] 12 purpose-bred adult research cats were enrolled. The cats were 3-year-old domestic short-haired cats of mixed sex (6 male castrated, 4 female spayed, and 2 female intact). Before enrollment, cats were maintained at a research facility and group-housed. They were fed ad libitum a commercially available adult dry diet (Meow Mix Original Choice, Big Heart Pet Inc, San Francisco, California) for at least 12-weeks before enrollment. The nutrient content of the commercial diet is found in Supplemental Information (Table S1). To determine health status, a physical examination, CBC, serum biochemistry panel, urinalysis, and total serum thyroxine concentration were performed in all cats before enrollment. The body weight, body condition score (BCS; Purina 1-9 point scale; Nestle Purina, St. Louis, Missouri), muscle condition score (MCS; 3, normal muscle mass; 2, mild muscle wasting; 1, moderate wasting; 0, severe wasting)[16-18] and select liver and kidney laboratory variables from the cohort of cats included in this study are summarized in Table 1. All cats had a normal physical examination with the exception of 1 intact female with a grade II/VI left parasternal heart murmur of unknown etiology and mild paraspinal muscle atrophy. Laboratory tests were normal for all cats including a creatinine <1.8 mg/dL and urine specific gravity >1.045. The cats were housed and cared for in accordance with a protocol that was approved by the Institutional Animal Care and Use Committee at the contract research facility that was used for the study (HQR protocol 170.045).

2.2 | Sample collection

For parts 1 and 2, feed was withheld for 12 hours and then cats were sedated with ketamine (20 mg IM per cat) and midazolam (10 mg IM per cat) for placement of jugular catheters. Aseptic placement of either 4 French 15-cm wire guided single lumen jugular catheter (MILACATH, MILA International, Inc, Florence, Kentucky; 10/12 cats) or single lumen catheter-through-cannula jugular catheter (Cavafix Certo, B Braun Medical AG, Melsungen, Germany; 2/12 cats) was placed in either the left or right jugular vein approximately 16 hours before the start of part 1 and 36 hours before the start of part 2. After catheter placement, all cats were housed in a separate kennel and

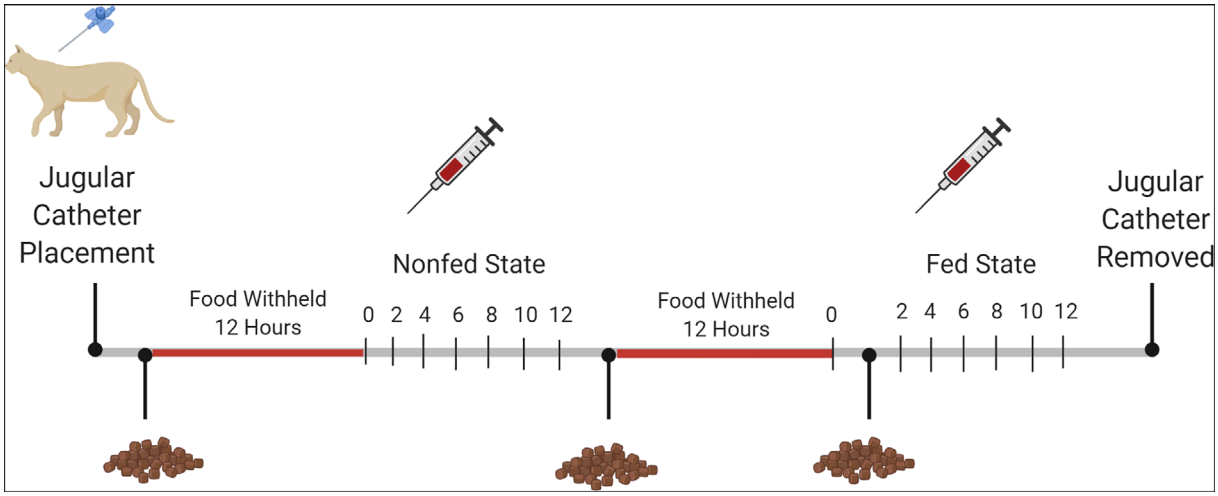

FIGURE 1 Schematic representing the timing of feeding and blood sampling for determining serum concentrations of serum indoxyl sulfate, p-cresol sulfate, and trimethylamine-n-oxide in healthy adult cats in the nonfed state (part 1) and fed state (part 2)

TABLE 1 Physical examination variables and the liver and kidney clinicopathologic variables for 12 study cats

Variable (reference interval)	Median (range)
Body weight (kg)	4.5 (3.0-6.8)
BCS (score 1-9)	5 (5-6)
MCS (score 0-3)	3 (2-3)
Creatinine (0.8-2.4 mg/dL)	1.4 (0.9-1.7)
BUN (18-35 mg/dL)	23 (17-27)
ALP (10-80 IU/L)	43 (34-64)
ALT (30-140 IU/L)	27 (14-50)
GGT (0-0.5 IU/L)	0 (0)
Total Bilirubin (0.0-0.1 IU/L)	0 (0-0.1)
Total thyroxine (1.2-4.8 μg/dL)	2.2 (1.7-4.2)
USG (≥1.035)	1.057 (1.049-1.064)

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; BCS, body condition score; BUN, blood urea nitrogen; GGT, gamma-glutamyl transferase; MCS, muscle condition score; USG, urine specific gravity.

provided a litterbox and water ad libitum. All cats recovered uneventfully from sedation. The catheters were secured and heparin locked (100 U/mL heparin sulfate).

For part 1, after food was withheld overnight for a 12-hour period a total of 7 blood samples were collected between 0600 (hour 0) and 1800 (hour 12) using a 3-syringe technique. For the 3-syringe technique, 1 mL of heparinized saline was flushed through the catheter and 3-mL of blood was aspirated into the syringe. The syringe with heparinized blood was detached, a second 2 to 3 mL of blood was collected using a 3-mL syringe (Monoject, Covidien, Dublin, Ireland), and unheparinized blood was placed into a 5-mL glass tube silicone-coated interior with no anticoagulants (Monoject Blood Collection Tube Red Stopper, Covidien). The heparinized blood was then returned to the cat and the catheter was flushed with heparinized saline. Blood samples (2-3 mL depending on body weight) were collected at 0600 (hour 0), 0800 (hour 2), 1000 (hour 4), 1200 (hour 6), 1400 (hour 8), 1600 (hour 10), and 1800 (hour 12). After the final sample collection, cats were offered 50 g of their diet for 1 hour in the PM. For part 2, after food was withheld overnight for a 12-hour period a blood sample (2-3 mL) was collected (0600; hour 0) and cats were offered 50 g of food for 1 hour. All cats ate 75% to 100% of the food. Blood samples (2-3 mL) were then collected at 0800 (hour 2), 1000 (hour 4), 1200 (hour 6), 1400 (hour 8), 1600 (hour 10), and 1800 (hour 12) using the same 3-syringe technique (Figure 1).

Sample collection and processing were standardized to minimize pre-CV_A. At each blood draw time, cats were collected in the same sequential order by 1 operator. Blood samples were centrifuged at 3000*g* for 10 minutes and the serum was aliquoted and then stored at 4°C for less than 12 hours before transferred to −80°C freezer. Jugular catheters were then removed without sedation and cats were moved to group-housing for the remainder of the study.

To determine medium-term biological variation, in part 3, blood samples were obtained 5 times over a 19-day period on days 0, 5, 8, 14, and 19 (3-6 day interval). After food was withheld overnight over a 12-hour period, each cat was sedated with an injection of ketamine (10-20 mg IV) and midazolam (5-10 mg IV). A blood sample (3 mL) was collected between 0800 and 0900 hours by venipuncture of either the left or right jugular vein from each cat using a 22-gauge needle and 3-mL syringe (Monoject, Covidien) by a single operator. After removal of the needle, blood was transferred into a 5-mL glass tube with silicone-coated interior and no anticoagulants (Monoject Blood Collection Tube Red Stopper, Covidien). Samples were centrifuged at 3000*g* for 10 minutes and the serum was aliquoted into 1.5-mL plastic vials. The serum samples for part 3 were at 4°C for less than 2 hours before placement in a −80°C freezer. Cats were sampled in the same sequential order on each collection date so that sampling occurred at approximately the same time of day for each cat.

Serum samples from parts 1 to 3 were stored at −80°C for 9 months before thawing and analysis at Colorado State University, Bioanalysis and Omics.

2.3 | Uremic toxin assay

Total IS, pCS, and TMAO serum concentrations were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Serum samples were prepared in 3 batches according to the 3 study parts (parts 1-3). Within each batch, samples were extracted in a randomized order and in duplicates. Each section batch, including its 2 replicates, was acquired within 1 acquisition batch in a randomized order different from its sample prep order. Details of the assay are found in the Supplemental document.

2.4 | Statistical analysis

Box and whisker plots were constructed (GraphPad Prism 9.0.0; GraphPad Software, La Jolla, California) for each toxin in part 1 (short-term) and part 3 (medium-term) to qualitatively describe variance between individuals (CV_G) and variance within individuals (CV_I). Statistical analysis was performed to determine biological variation estimates according to previously reported guidelines using a specialized statistical software (SAS, Version 9.4; SAS Institute, Cary, North Carolina).[12]

Residual diagnostic plots were used to evaluate model assumptions of normality and equal variance. Because of skew and unequal variance, a (natural) log transformation was used for each toxin in each study part to better satisfy model assumptions. Log transformed data were assessed for outliers using a 3-step process. Data were assessed for outliers by evaluating values falling outside 3 times the interquartile range (a) across all cats, (b) within each cat, and (c) using cat level averages (where a single average was calculated for each toxin, study part and cat). For part 1, no outliers were identified. For pCS in part 2, a single time point for a single cat (both duplicates) was identified as an outlier. For pCS and TMAO in part 3, a single time point for a single cat (both duplicates) was identified as an outlier. All outliers were identified when looking "within cat" but not at the other levels. All observations were retained in the analysis because the outlying values were similar between duplicates and thus were unlikely to be because of analytical error.

A random effects model was fit using restricted maximum likelihood (REML) with SAS Proc Mixed. Cat and time point (nested within cat) were included in the model as random effects. Hence, the variance was partitioned into 3 components for each toxin: (a) CV_G, (b) CV_I, and (c) variation between duplicates (CV_A).

Because the analysis was done on the log transformed scale, the (back transformed) coefficients of variation (CV) were calculated, as previously described,[19,20] using the equation:

$$CV = \sqrt{(\exp(\sigma^2) - 1)}$$

The II was calculated from the CVs using the formula[12]:

$$II = \frac{CV_G}{\sqrt{CV_I^2 + CV_A^2}}$$

Because the analysis was done on the log transformed scale, RCVs were calculated using the lognormal approach, as previously described.[19,20] Specifically:

TABLE 3 Reference change values for serum indoxyl sulfate, p-cresol sulfate, and trimethylamine-n-oxide (TMAO) in healthy adult cats

Analyte	Reference change value (%)	
	Decrease	Increase
	Short-term biological variation (n = 10)	
Indoxyl sulfate	44.6	224.4
p-Cresol sulfate	33.1	302.1
TMAO	56.6	176.6
	Medium-term biological variation (n = 12)	
Indoxyl sulfate	21.9	455.7
p-Cresol sulfate	28.9	345.8
TMAO	52.2	191.7

TABLE 2 Biological variation for indoxyl sulfate, p-cresol sulfate, and trimethylamine-n-oxide (TMAO) expressed as coefficients of variation for group (or between cat variation; CV_G), individual variation (CV_I), and analytical variation (CV_A) and the index of individuality (II)

Analyte	Median (range)	CV_G (%)	CV_I (%)	CV_A (%)	II
	Short-term variation (n = 10)				
Indoxyl sulfate	363 (90-1541 ng/mL)	58.2	29.7	2.2	1.96
p-Cresol sulfate	10 958 (3013-46 666 ng/mL)	40.7	41.4	3.0	0.98
TMAO	828 (373-1457 ng/mL)	30.4	20.6	2.3	1.47
	Medium-term variation (n = 12)				
Indoxyl sulfate	494 (101-1917 ng/mL)	38.7	59.0	2.5	0.65
p-Cresol sulfate	19 328 (3266-77 609 ng/mL)	55.0	47.0	2.9	1.17
TMAO	888 (375-1507 ng/mL)	19.7	23.7	2.7	0.83

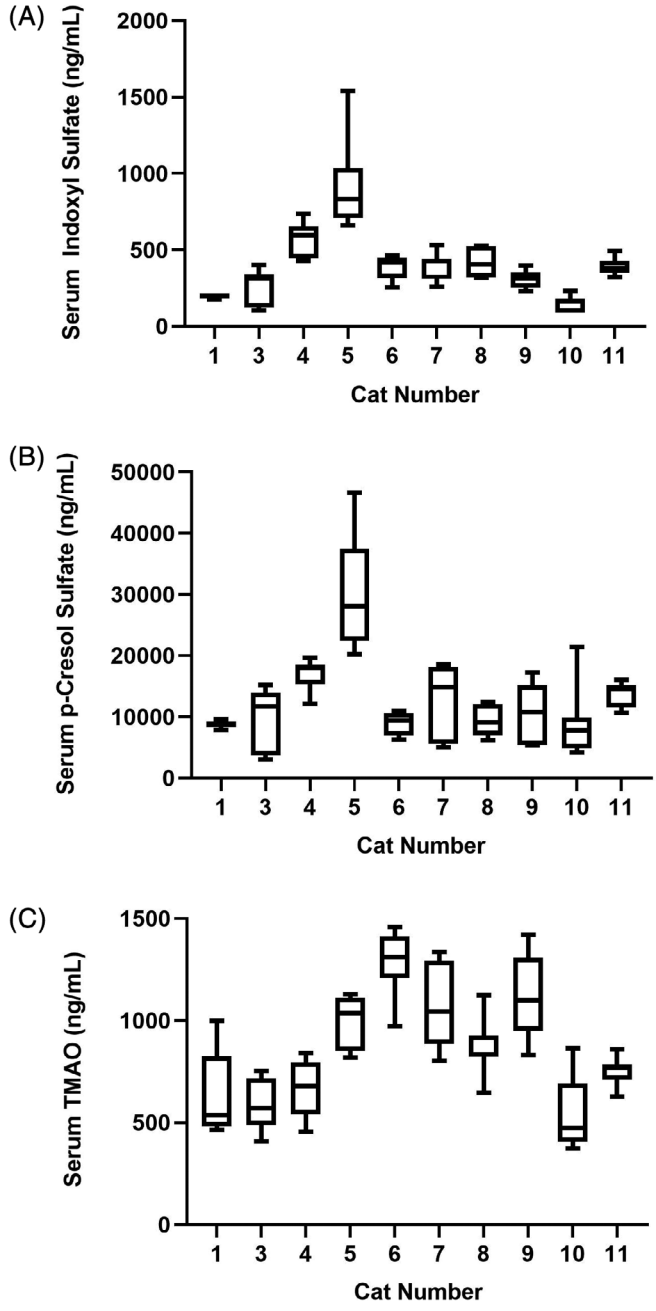

FIGURE 2 Box and whisker plots for serum indoxyl sulfate (A), p-cresol sulfate (B), and trimethylamine-n-oxide (TMAO; C) concentrations in 10 healthy adult cats sampled every 2 hours over a 12-hour period in a nonfed state. Samples could not be obtained from cat 2 and cat 12 because of jugular catheter malfunction. Boxes represent the interquartile range from the 25th to 75th percentile. The T-bars (whiskers) show the range of the individual cat's data

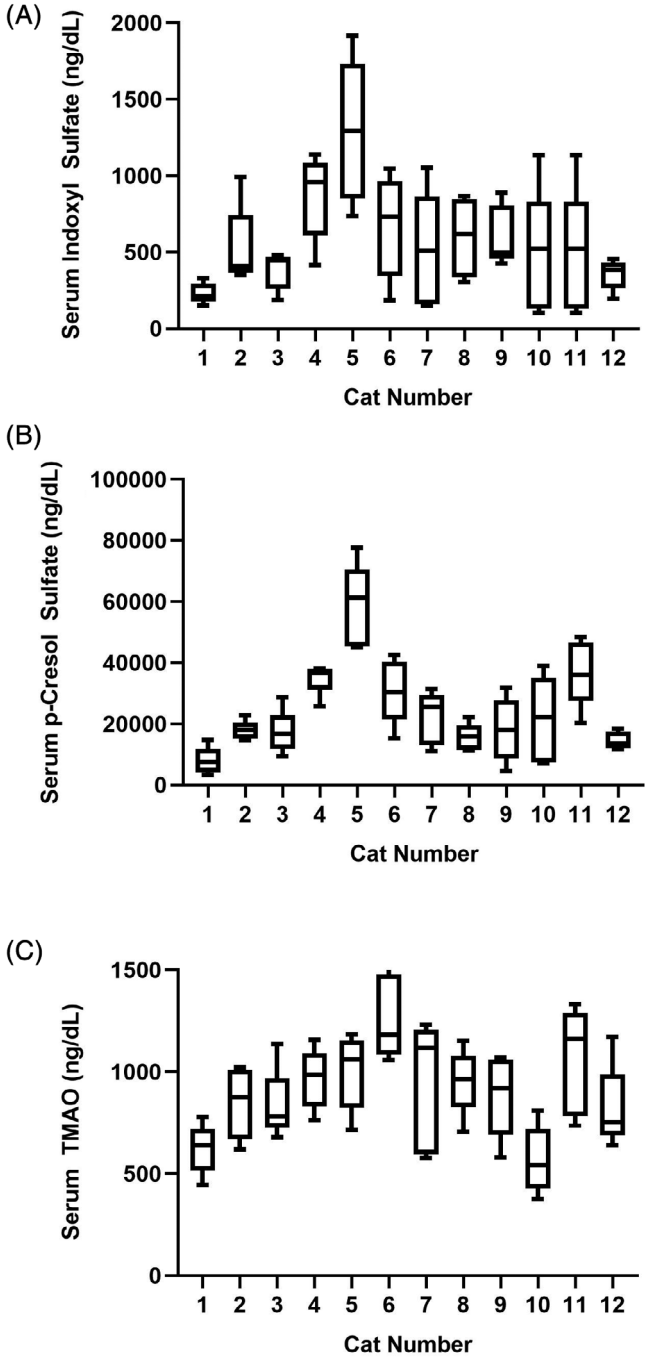

FIGURE 3 Box and whisker plots for serum indoxyl sulfate (A), p-cresol sulfate (B), and trimethylamine-n-oxide (TMAO; C) concentrations in 12 healthy adult cats sampled 5-times over a 19-day period in a nonfed state. Boxes represent the interquartile range from the 25th to 75th percentile. The T-bars (whiskers) show the range of the individual cat's data

$$\mathrm{RCV}(\%) = \exp\left(\pm Z\sqrt{2\times\left(\sigma_I^2+\sigma_A^2\right)}\right)$$

where Z = 1.96 for 2-sided interpretation and σ^2 represents a log scale variance component.

To determine if recent feeding should be considered a variable when assessing serum concentrations collected at the same time of day, serum IS, pCS, and TMAO concentrations (from only cats that completed both part 1 and part 2 of the study; n = 9) were compared between the fed state and nonfed state at hours 2, 4, 6, 8, 10, and 12 using a paired *t* test or Wilcoxon matched-paired signed rank test. To determine whether the time of day should be considered a variable when assessing serum concentrations, a repeated measures 1-way

ANOVA with the Geisser-Greenhouse correction followed by a Tukey's post hoc analysis or a Friedman test with Dunn's post hoc analysis was used to compare serum IS, pCS, and TMAO concentrations between different time points over a 12-hour period in the nonfed state (hours 0, 2, 4, 6, 8, 10, 12) and over a 10-hour period in the fed state (hours 2, 4, 6, 8, 10, 12). The average of the duplicates taken at each time point for each cat was used in the analysis. Data were log-transformed to base-10 to meet the assumption of normality. If normality was not met then a nonparametric test was performed on the raw values. Statistical analysis was performed using a statistical software program (GraphPad Prism 9.0.0; GraphPad Software). A *P*-value <.05 was considered significant.

3 | RESULTS

Of the 12 enrolled cats, 10 cats finished part 1 and 9 cats finished part 2 with the other cats removed because of catheter malfunction or displacement. All 12 cats completed part 3 of the study. In the 3 study parts, CV_A was less than 25% of within individual variation ($CV_A < 0.25 \times CV_I$) for IS, pCS, and TMAO analytes indicating optimal precision.[21] In general, the coefficients of variations between cats (CV_G) and within individual cats (CV_I) were large for the 3 toxins (approximately 20%-60%). Values of CV_G, CV_I, CV_A, and indices of individuality for serum IS, pCS, and TMAO concentrations in healthy adult cats from study parts 1 and 3 are reported in Table 2. The RCVs for serum IS, pCS, and TMAO concentrations are reported in Table 3.

For serum IS concentrations over a 12-hour period (Figure 2A), the variation between cats (CV_G) was greater than the within-cat variation (CV_I) corresponding to a high II and supporting the use of a subject-based reference interval. On the contrary, the CV_I for serum IS concentrations over a 19-day period (Figure 3A) was greater than CV_G corresponding to a low II. Therefore, a population-based reference interval is appropriate in this clinical scenario. For serum pCS concentrations, the CV_G and CV_I were similar over a 12-hour period (Figure 2B). Over a 19-day period, serum pCS concentrations had greater CV_G when compared to those in CV_I (Figure 3B). For serum TMAO concentrations, the CV_G was greater than CV_I over a 12-hour period (Figure 2C) and the contrary was found over a 19-day period (Figure 3C). For serum pCS and TMAO concentrations, the II was intermediate using both short-term and medium-term biological variation estimates supporting the use of a population-based reference interval in conjunction with a subject-based reference interval depending on the clinical picture.

Serum IS (Figure 4A), pCS (Figure 4B), and TMAO (Figure 4C) concentrations were significantly lower in the fed state when compared to the nonfed state at several time points throughout the 12-hour period. Serum IS concentrations were significantly lower in the fed state when compared to the nonfed state at hour 4 (*P* = .006), hour 6 (*P* = .005), hour 8 (*P* = .05), and hour 12 (*P* = .03; Figure 4A). Serum pCS concentrations were significantly lower in the fed state when compared to the nonfed state at hour 4 (*P* = .004) and Hour 6 (*P* = .008; Figure 4B). Serum TMAO concentrations were significantly lower in the fed state when compared to the nonfed state at hour 2 (*P* = .007), hour 4 (*P* < .001), hour 6 (*P* < .001), hour 8 (*P* < .001), hour 10 (*P* < .001), and hour 12 (*P* < .001; Figure 4C).

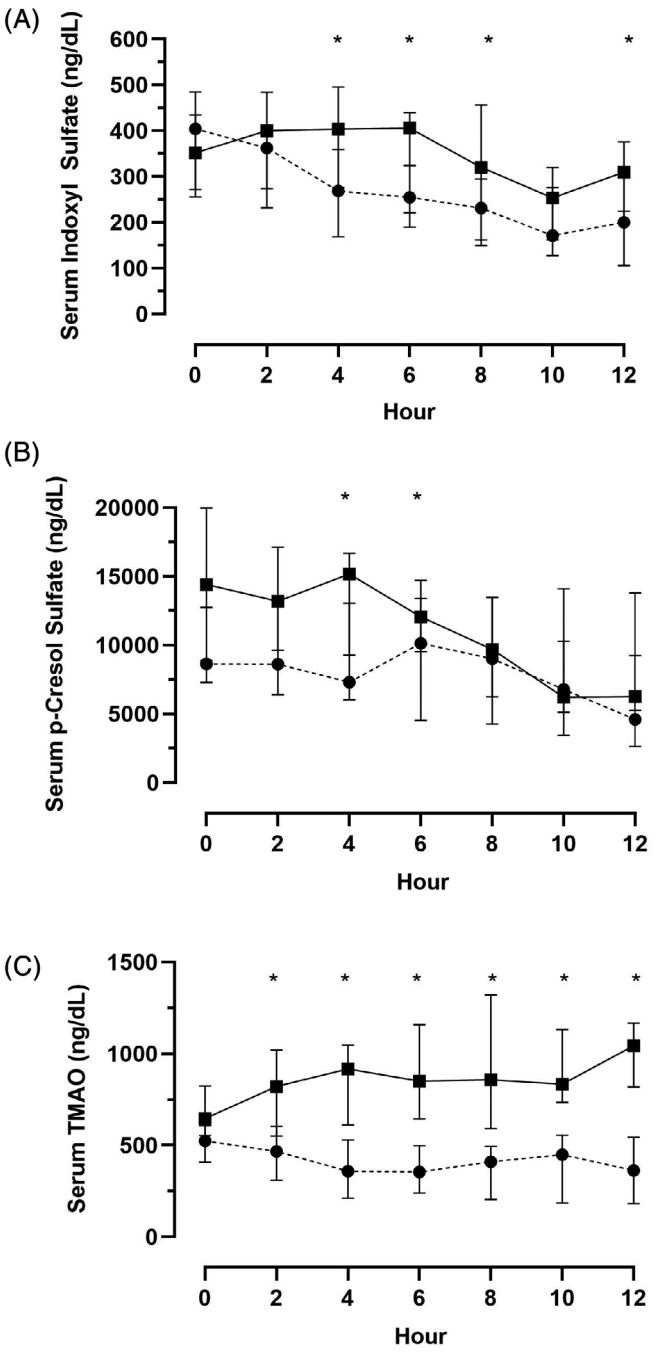

FIGURE 4 Serum indoxyl sulfate (A), p-cresol sulfate (B), and trimethylamine-n-oxide (TMAO; C) concentrations (median and interquartile range) in 9 healthy adult cats over a 12-hour period. Serum was collected after food was withheld for a 12-hour period (hour 0) and then every 2 hours in a nonfed state (square with solid line) and after a meal (circle with dotted line) at hours 2, 4, 6, 8, 10, 12. Asterisk means a significant (*P* < .05) difference was found between the fed and nonfed state at that hour

In the nonfed state, serum pCS concentrations were significantly different at hour 2 compared to hour 12 (*P* = .03), at hour 4 compared to hour 10 (*P* = .01) and hour 12 (*P* < .001), hour 6 compared to hour

12 (P = .02), and hour 8 compared to hour 12 (P = .04). Serum IS and TMAO did not significantly differ between time points throughout a 12-hour period in the nonfed state. In the fed state, serum IS concentrations were significantly different at hour 2 compared to hour 8 (P = .04), hour 10 (P = .02), and hour 12 (P = .01) and at hour 6 compared to hour 8 (P = .01). Serum concentrations of pCS and TMAO did not significantly differ between time points in the fed state.

4 | DISCUSSION

Our study determined the short-term and medium-term biological variation estimates for serum IS, pCS, and TMAO concentrations in healthy adult cats. In general, serum IS, pCS and TMAO concentrations have large intra- and interindividual variability. Although study design varies among studies, our findings are relatively similar to studies in healthy people. One study evaluated the medium-term biological variation of total IS and pCS based on 8 samples collected daily on consecutive days in 10 healthy individuals. In this study,[14] the CV_I and CV_G were 36% and 25% for total IS and 51% and 64% for total pCS, respectively. The CV_I and CV_G are also high for TMAO in healthy people and in people on hemodialysis.[15,22,23] These large variations has been attributed to the fact that the 3 uremic toxins are end-products of protein catabolism in the colon. Urea, another product of protein catabolism, also has large intra- and interindividual variation in cats.[20,24] In people, this notable variability in the major uremic toxins was assumed to be because of the changes in nutritional intake; however, this is likely not the case because in our study the cats were on the same diet and samples were collected after food was withheld. Other physiologic factors that might affect serum concentrations of IS, pCS, and TMAO are not well understood. Renal function, age, and sex are independent determinants of serum IS and pCS concentrations in people and host heritability also affect concentrations.[25] This is likely because of, at least in part, the role these factors play in shaping the composition of the gut microbiome and therefore their impact on amino acid processing in the intestines.[1,26,27]

Serial monitoring of serum IS, pCS, and TMAO concentrations could be beneficial in cats, especially those with CKD, to help determine disease stage, risk of disease progression, and efficacy of dietary intervention. In most cases, veterinarians and researchers would be monitoring serial measurements with >24 hours between sample collections, therefore the RCVs using medium-term biological variation estimates would be most appropriate in this scenario. The RCV is the percent change (either decrease or increase) between sequential measurements that suggest a relevant change beyond random variation. Importantly, the RCV is calculated based on CV_I for the analyte and CV_A for the instrument upon which the specimens were analyzed; therefore, an RCV value is not universal. An RCV is used to calculate a subject-based reference interval by applying the percent RCV to the mean analyte concentrations taken from an individual, ideally during health. An RCV can also be used to decide if a change between 2 measurements is significant. In our study, the RCV for IS, pCS, and TMAO based on the medium-term biological variation estimates suggests that serum concentrations would have to decrease by 21.9%, 28.9%, and 52.2%, respectively, between serial measurements to support a significant biological change. For example, a cat had a previous TMAO concentration of 300 ng/dL. A decrease to 120 ng/dL (a decrease of 60%) in a subsequent sample 2 weeks later represents a decrease greater than the RCV of 52.2%; therefore, the change is likely a true relevant biological change. The RCV for an increase in serum concentration was large for IS (455.7%), pCS (345.8%), and TMAO (191.7%). Because of this finding, it seems these uremic toxins would be best utilized to monitor treatment effect (ie, reduction in serum concentrations) rather than prognostic biomarkers (ie, monitoring for increases in serum concentrations). However, to date no published studies evaluating the utility of serial monitoring of serum uremic toxin concentrations in cats are available. The biological variation data from this study will be helpful in future studies evaluating the potential utility of using serum IS, pCS, and TMAO concentrations as prognostic or therapeutic biomarkers.

The II is used to determine whether a population-based or subject-based reference interval is more appropriate when monitoring serial measurements for an analyte. For an analyte with a low degree of individuality (<0.7), a population-based reference interval can be used to detect clinical disease. If an analyte has a high degree of individuality (≥1.7), then the RCV is more sensitive to determine if a change in an analyte concentration for an individual cat is clinically significant. For analytes with intermediate individuality (II 0.7-1.69), a population-based reference interval can be used; however, a subject-based reference interval could detect mild changes in the health status of an individual.[12,19] Using the II for IS, pCS, and TMAO using medium-term biological variation estimates, a population-based reference interval can be used to monitor for significant changes in the serum concentrations; however for pCS and TMAO, the veterinarian should also refer to the subject-based reference intervals to detect subtle changes in serum concentrations for an individual cat; for example, a cat with a serum pCS concentration just above the population-based reference interval. To date, a population-based reference interval for serum IS, pCS, and TMAO has not been determined. In the meantime, the CV_I from our study and the CV_A for the instrument upon which the samples are analyzed can be used to calculate an RCV to be applied to future measurements in either a research or clinical setting to determine a clinically important change in the analyte concentration.

Because IS, pCS, and TMAO are derived from dietary protein, we hypothesized that serum concentrations would increase after a meal in cats. A study in healthy human adults observed that serum concentration of IS increased significantly after meals, whereas serum pCS concentrations did not exhibit postprandial changes.[28] To the authors' knowledge, no such information is published on serum TMAO concentrations in people. Indoxyl sulfate, pCS, and TMAO are end-products of amino acid fermentation in the colon and exclusively originate from the intestinal tract.[29,30] Dietary protein is digested into peptides in the stomach and small intestine. A portion of undigested dietary proteins and peptides enter the colon and are depolymerized by bacterial proteases and peptidases into small oligopeptides and amino acids. In the distal colon, the amino acid tryptophan is fermented to indoles by gut microbiota that express the tryptophanase

enzyme, and the amino acids phenylalanine and tyrosine are fermented to p-cresol by anaerobic gut microbiota. Indole and p-cresol are absorbed into systemic circulation, sulfated in the liver to IS and pCS, respectively, and reversibly bind to plasma albumin.[30-32] For TMAO, gut microbiota synthesize trimethylamine from the amino acids choline and L-carnitine. In circulation, trimethylamine is oxidized to TMAO in the liver, and this toxin is not protein bound.[30,33-35] Preformed TMAO can also be found in certain foods, particularly fish, and can be directly absorbed from the gut.[36] Indoxyl sulfate and pCS are eliminated by organic anion transporter-mediated proximal tubular secretion, and TMAO is eliminated by glomerular filtration.[35,37] Serum concentrations of these major gut-derived uremic toxins are therefore affected by diet composition, gut microbial composition, and liver and kidney function.[2,6,8,32,38]

We found that serum concentrations of IS, pCS, and TMAO were often significantly lower in the fed state when compared to the nonfed state at multiple time points over a 12-hour period in cats, thus rejecting our hypothesis. This is in contrast to findings in humans, however the exact time frame in which serum concentrations of IS were found to be elevated after a meal is unclear so direct comparison is challenging.[28] The reason for lower serum concentrations in the fed state compared to the nonfed state in healthy cats is unknown. To the best of our knowledge, the time it takes for microbial byproducts to be produced, absorbed, and sulfated by the liver is unknown, and is likely subject to individual variation. However, as total gastrointestinal transit time is over 12 hours in young cats (26.5 ± 5.8 hours),[39] the protein in the food at mealtime was unlikely contributing to the changes in serum concentrations noted during the 12-hours after feeding. Many environmental factors as well as age and the diet were controlled in our study. The only factors that differed between parts 1 and 2 were the day the samples were collected, the timing of sample collection after feeding (ie, fed vs nonfed state), and the sample batch during analysis. Considering these factors, we postulate that the lower serum concentrations in the fed state might be because of day-to-day variation in intestinal transit time or intestinal absorption of microbial metabolic byproducts or physiologic variation in renal tubular excretion. Another possible explanation for lower postprandial serum concentrations is the stimulation of colonic motility by the gastric-colic reflex after a meal and reduced colonic transit time. A potential batch effect during analysis is unlikely considering the use of internal standard and calibration curves.

For IS and pCS, serum concentrations gradually declined over a 12-hour period in both the fed and nonfed state. Although we did not evaluate serum concentrations over a 24-hour period, this finding supports a possible circadian rhythm of serum IS and pCS concentrations. This is a similar finding in healthy people.[14] When interpreting variance of metabolites using biological variation estimates, it is assumed that concentrations are relatively stable over time. Because a trend over time was appreciated in our study, the short-term biological variation coefficients of variance should be interpreted with some caution.

We found that serum concentrations of IS and pCS vary depending on the time of day. On the contrary, serum TMAO concentrations were relatively static over the 12-hour period in both the fed and nonfed state (Figure 4). In general, serum IS and pCS concentrations were different in the morning (0600-1000; hours 0-4) when compared to the late afternoon (1400-1800; hours 8-12). This suggests that in order to compare serum concentrations of IS and pCS between 2 time points in an individual cat, the serum sample should be collected at relatively the same time of day.

In human medicine, it has become common practice to apply biological variation values, including RCV and CV_I, from healthy people to those with chronic disease. This is an appropriate strategy in many chronic diseases, but not all.[40] Protein malassimilation occurs in people with CKD allowing higher amounts of protein to enter the colon which likely contributes to the variability in serum concentrations of IS, pCS, and TMAO between people with CKD.[6,41,42] A similar phenomenon might be present in cats with CKD as a study demonstrated overlapping serum concentrations of IS and pCS between the stages of disease.[38] It is plausible that variability in dietary protein consumption, protein malassimilation, and reduced renal elimination exaggerates serum concentrations in cats with CKD. Thus, determination of biological variation values for serum concentrations of IS, pCS, and TMAO in cats with CKD is warranted to assess the clinical significance of changes between serial laboratory values in this specific disease population.

Our study had limitations. Although the health status of the cats were thoroughly evaluated before enrollment, it is difficult to unequivocally prove health status and especially the absence of early kidney dysfunction. Imaging of the urinary tract and glomerular filtration rate determined by iohexol clearance or nuclear scintigraphy were not performed in these cats which can be used to detect early kidney dysfunction in cats. Second, varying sampling intervals ranging from 3 to 6 days were used to determine medium-term biological variation in our study. According to Freeman et al,[12] initial studies of biological variation should use a standardized interval of 7 days between collections. In particular, greater duration between sample collections results in higher variation (most notably, CV_I). Although variability tends to stabilize after 3 to 4 days, variable sampling intervals will give variable CVs making it unclear regarding what time period the variation applies to.[43-45] The dates of sample collection used to determine medium-term variation in our study was based on the availability of support staff for sample collection and cat availability. Third, cats were sedated using a standard protocol (ketamine and midazolam) before collection of the serum samples used to determine medium-term variation. This protocol is unlikely to be required for sample collection in most clinical cases and could have affected the results to an unknowingly degree. Fourth, research cats were chosen for the study rather than healthy, young client-owned cats to minimize interindividual variability because of homogeneity of living conditions and diet. Fifth, the serum aliquots were stored in a −80°C freezer for 9 months before analysis. Although it has been demonstrated that TMAO is stable for up to 5 years in storage at −80°C,[10] to our knowledge no study has evaluated the stability of serum IS and pCS stored at −80°C beyond 3 months.[9] The sample storage conditions in this study could have altered the serum measurements to an unknown degree. Sixth, it is unknown whether the results of the study apply to cats with pathology. Therefore, caution should be applied when applying results to client-owned cats or cats with disease, specifically CKD.

Our study provides biological variation estimates for the major gut-derived uremic toxins IS, pCS, and TMAO in serum. In conclusion, the results of this study demonstrate intermediate individuality for serum pCS and TMAO analytes for both short- and medium-term biological variation estimates supporting the use of a population-based reference interval with reference to the subject-based reference interval. Serum IS analyte had high individuality for short-term biological variation estimates and low individuality for medium-term biological variation estimates supporting the use of a subject-based reference interval and a population-based reference interval, respectively. Feeding might reduce serum concentrations of pCS, IS, and TMAO over a 12-hour period in cats. To compare serial measurements, it would be prudent to collect samples at the same time of day and consistently in either a fed or nonfed state.

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CONFLICT OF INTEREST DECLARATION

Authors declared no potential conflicts of interest. Drs Summers, Quimby, and Lappin are paid consultants of Nestle Purina Pet Care; however, this project is unrelated to their consultation agreement.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

The cats were housed and cared for in accordance with a protocol that was approved by the IACUC at the contract research facility that was used for the study (HQR protocol 170.045).

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.

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