Journal of Veterinary Internal Medicine
Open Access

J Vet Intern Med 2015;29:597–602

Urinary and Plasma Catecholamines and Metanephrines in Dogs with Pheochromocytoma, Hypercortisolism, Nonadrenal Disease and in Healthy Dogs

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Background: Diagnosis of pheochromocytoma (PC) is based on a combination of clinical suspicion, finding an adrenal mass, increased plasma, and urine concentrations of catecholamine metabolites and is finally confirmed with histopathology. In human medicine, it is controversial whether biochemically testing plasma is superior to testing urine.

Objectives: To measure urinary and plasma catecholamines and metanephrines in healthy dogs, dogs with PC, hypercortisolism (HC), and nonadrenal diseases (NAD) and to determine the test with the best diagnostic performance for dogs with PC.

Animals: Seven PC dogs, 10 dogs with HC, 14 dogs with NAD, 10 healthy dogs.

Methods: Prospective diagnostic clinical study. Urine and heparin plasma samples were collected and stored at −80°C before analysis using high-pressure liquid chromatography (HPLC) coupled to electrochemical detection or tandem mass spectrometry were performed. Urinary variables were expressed as ratios to urinary creatinine concentration.

Results: Dogs with PC had significantly higher urinary normetanephrine and metanephrine : creatinine ratios and significantly higher plasma-total and free normetanephrine and plasma-free metanephrine concentrations compared to the 3 other groups. There were no overlapping results of urinary normetanephrine concentrations between PC and all other groups, and only one PC dog with a plasma normetanephrine concentration in the range of the dogs with HC and NAD disease. Performances of total and free plasma variables were similar. Overlap of epinephrine and norepinephrine results between the groups was large with both urine and plasma.

Conclusion and clinical importance: Measurement of normetanephrine is the preferred biochemical test for PC and urine was superior to plasma.

Key words: Canine; Diagnosis; Hyperadrenocorticism; Mass spectrometry.

Pheochromocytomas (PC) are catecholamine-producing tumors arising from chromaffin cells of the adrenal medulla.[1] Clinical signs result most often from secretion of excessive amounts of catecholamines, and rarely from the space-occupying or invasive nature of the tumor.[2–8] Diagnosis of PC in humans is mainly based on biochemical detection of catecholamine-derived secretory products. Commonly used tests are measurement of catecholamines and their O-methoxylated metabolites metanephrines (normetanephrine and metanephrine) in 24-h urine samples or in plasma.[1,9–12] The question of whether urine or plasma is best is still somewhat controversial, but plasma metanephrines are recommended more often as test of choice.[11–16] In dogs, evaluation of those biomarkers for the diagnosis of PC only started a few years ago; dogs with PC, but also those with nonadrenal illness and dogs with hypercortisolism (HC) can have increased catecholamines and metanephrines excretion.[17–21]

Abbreviations:

AT	adrenocortical tumor
HC	hypercortisolism
HPLC	high-pressure liquid chromatography
LDDS	low-dose dexamethasone suppression test
NAD	nonadrenal disease
PDH	pituitary-dependent hypercortisolism
PC	pheochromocytoma

PC cannot be distinguished ultrasonographically from tumors of the adrenal cortex and there are several clinical signs of PC and HC that overlap, which makes HC an important differential diagnosis. Therefore, both, dogs with HC and nonadrenal disease (NAD) are important controls in the evaluation of biomarkers for the diagnosis of PC.

Since 24-h urine sampling is impracticable, measurements of urinary fractionated catecholamines and metanephrines was established in spot urine samples by expressing their concentrations as a ratio to the urinary creatinine concentration.[19] The urinary normetanephrine to creatinine ratio was shown to be the parameter

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This study was presented in part as an oral abstract at the Congress of the European College of Veterinary Internal Medicine - Companion Animals, Maastricht, Netherlands 2012.

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Submitted January 26, 2014; Revised November 27, 2014; Accepted February 3, 2015.

DOI: 10.1111/jvim.12569

which differentiated dogs with PC best from healthy dogs and dogs with HC.[20,21] Both, free normetanephrine and free metanephrine in plasma parameters were significantly higher in dogs with PC compared to healthy dogs, dogs with adrenocortical tumors (AT), and dogs with NAD; of all tested variables, plasma-free normetanephrine discriminated best between diseases.[18]

No comparison between urinary and plasma variables has been carried out in dogs.

The aim of this study was to measure urinary and plasma catecholamines and metanephrines in healthy dogs, dogs with PC, HC, and NAD and to determine the test with the best discrimination for dogs with PC. With regard to the plasma tests, an additional aim was to compare the diagnostic performance of total (free + sulfoconjugated) and free metanephrines.

Materials and Methods

Animals

Dogs with Pheochromocytoma. Seven dogs with PC were included; 4 of them were male (2 castrated) and 3 were spayed females. Age ranged between 8.9 and 13 years (median 11) and body weight between 4.1 and 22.8 kg (median 11.5). Breeds included Jack Russell Terrier (1), West Highland White Terrier (1), Fox Terrier (1), Cairn Terrier (1), Standard Schnauzer (1), Papillon (1), and 1 mixed-breed dog. Dogs were prospectively enrolled if diagnosis was confirmed based on histopathologic examination (4 dogs) of the adrenal gland, an increased urinary normetanephrine to creatinine ratio above the previously established cut-off of 4 times the upper range of normal (3 dogs), or both.[19]

At presentation dogs showed various clinical signs indicative of PC, including weakness, panting, tachycardia, agitation, trembling, polyuria/polydipsia, abdominal pain, and signs of gastrointestinal disease. By means of ultrasonography unilateral adrenal enlargement was identified in 5 dogs (right adrenal gland in 3 dogs, left adrenal gland in 2 dogs), in 2 dogs bilateral adrenal enlargement was found. In 4 dogs, diagnosis was confirmed by histologic examination after adrenalectomy or by postmortem examination. Two of these dogs were the dogs with bilateral adrenal enlargement. Histologic examination revealed unilateral PC and contralateral adrenocortical adenoma and bilateral adrenocortical hyperplasia, respectively. The latter dog had a positive low-dose dexamethasone suppression test (LDDS) and an endogenous ACTH concentration within the reference range and was considered to be suffering from concurrent pituitary-dependent hypercortisolism (PDH). In the dog with PC and concurrent contralateral adrenocortical adenoma, no work up for HC had been performed. One dog that had been presented with clinical signs highly suspicious for PC, a left-sided adrenal mass and increased U-normetanephrine : creatinine (497), finally was excluded, as no histopathologic examination of the adrenal gland and no follow-up information was available to confirm the diagnosis.

Dogs with Hypercortisolism. Ten dogs with HC were included; 6 dogs were male (5 castrated) and 4 were female (3 spayed). Age ranged between 6 and 15 years (median 10.5) and body weight between 7 and 40.5 kg (median 17.9). Breeds included Labrador Retriever (1), Nova Scotia Duck Tolling Retriever (1), Dalmatian (1), Yorkshire Terrier (1), Welsh Terrier (1), and 5 mixed-breed dogs.

Dogs were prospectively enrolled if clinical signs were consistent with HC (e.g., polyuria, polydipsia, polyphagia, panting, skin problems, weakness, abdominal enlargement), the LDDS test yielded a positive result and owners were ready to treat and regularly re-evaluate the dog over at least a 6-month period. PDH was diagnosed in 5 dogs by means of a normal or increased concentration of endogenous ACTH, bilateral symmetrical appearance of the adrenal gland determined by ultrasonography, demonstration of pituitary enlargement by computer tomography, or by both the methods. All 5 dogs with PDH underwent treatment with trilostane.[a] To minimize the risk that concurrent PC was present, as no histopathology of the adrenal glands was available, only those dogs with well-controlled PDH and no other clinical signs during a minimum of 6 months after starting treatment with trilostane were used in the study, according to our previous study design.[21,22]

HC caused by an adrenocortical tumor (AT) was suspected in 5 dogs on the basis of a low endogenous ACTH concentration and the finding of an adrenal mass by ultrasonography. AT was confirmed by histologic examination of the adrenal glands after adrenalectomy and resolution of clinical signs (3 dogs) or postmortem examination (2 dogs). All these dogs had unilateral adrenocortical carcinomas (right adrenal gland in 3 dogs and left adrenal gland in 2 dogs) on histological examination. Two dogs were excluded from the study, as either concurrent plasma and urine samples were not available, or HC was not diagnosed intravitam (no hormonal tests performed). In one of the dogs, a nodular hyperplasia of the adrenal glands and in the other canine hyperplasia of the left adrenal and a cortical adenoma of the right adrenal gland could be confirmed on histopathology. U-normetanephrine : creatinine were 146 and 97, respectively in these 2 dogs.

Dogs with Nonadrenal Disease. Fourteen dogs with NAD were included; 9 dogs were male (5 castrated) and 5 dogs were female spayed. Age ranged between 3 and 14 years (median 7) and body weight between 4.1 and 45 kg (median 23.8). Breeds enclosed Flat Coated Retriever (1), Labrador Retriever (1), Manchester Terrier (1), Border Terrier (1), Yorkshire Terrier (1), West Highland White Terrier (1), Alaskan Malamute (1), Dachshund (1), Standard Poodle (1), and 5 mixed-breed dogs. The dogs were suffering from diabetes mellitus (2), gastroenteritis (2), hepatic disease (2), gastrointestinal foreign body (1), chronic urinary tract infection (1), lung fibrosis (1), hypothyroidism and hemangiosarcoma (1), polyarthritis (1), dirofilariasis (1), garbage intoxication (1), idiopathic megaesophagus, and aspiration pneumonia (1). All dogs underwent complete workup including physical examination, complete blood count, serum biochemistry profile, urinalysis, urine culture, as well as diagnostic imaging. In all dogs, adrenal glands were either found to be normal on ultrasonography or they showed a normal suppression of cortisol in the LDDS test (or combination of both).

Healthy Dogs. Ten healthy client-owned dogs including 5 males (3 castrated) and 5 females (3 spayed) with a median body weight of 26.5 kg (range, 14.6– 59.2 kg) and a median age of 5.0 years (range, 2.0 – 7.0 years) were used. Breeds included Bernese Mountain Dogs (2), Border Collie (1), Labrador Retriever (1), Golden Retriever (1), Nova Scotia Duck Tolling Retriever (1), Rhodesian Ridgeback (1), and 3 mixed-breed dogs. They were determined to be healthy on the basis of history and results of physical examination, CBC, serum biochemical profile, and urinalysis including urine culture. None of the dogs had received any medication for at least 8 weeks before inclusion in the study except routine vaccination, deworming and heartworm prophylaxis.

All dogs were prospectively enrolled in the study according to the study protocol, which was approved by the veterinary office of the canton of Zurich and was in accordance with the guidelines and directives established by the Animal Welfare Act of Switzerland.

Sample Collection and Processing

Urine and plasma samples for the analysis of catecholamines and metanephrines were taken within 30 minutes during work up in the hospital. Urine collection and processing was done as

reported previously.[19–21] In brief, 10 mL of urine were placed in a plain silicone-coated tube containing 280 μL of 20% hydrochloric acid (HCl). Urinary pH was measured using pH indicator stripes (range of pH 1 – 6) and HCl was added to achieve a pH ≤2 if needed. Samples were light-protected and stored at −80°C until analysis. Plasma samples were collected in chilled heparin tubes, centrifuged at 4°C, snap frozen in liquid nitrogen and stored at −80°C. Samples were shipped on dry ice to the respective laboratory and thawed immediately before analysis.

Measurement of Catecholamines and Metanephrines

Urine samples were analyzed at the Institute of Clinical Chemistry, University Hospital, Zurich, Switzerland as previously described.[19–21] Urinary norepinephrine, epinephrine, total normetanephrine, and total metanephrine were quantified by HPLC with amperometric detection as separate compounds. The term "total" is used for the sum of free and conjugated metanephrine according to Grouzmann et al.[12] The term "metanephrines" (plural form) includes normetanephrine and metanephrine. The results are expressed as a ratio to urinary creatinine concentrations as reported previously[19–21] and will be listed in the following sections as: U-norepinephrine : creatinine, U-epinephrine : creatinine, U-normetanephrine : creatinine, and U-metanephrine : creatinine.

Plasma samples were analyzed in the laboratory of the Division of Clinical Pharmacology and Toxicology, University Hospital, Lausanne, Switzerland. Plasma norepinephrine and epinephrine were determined by HPLC with amperometric detection.[23] Plasma-free normetanephrine and free metanephrine were quantified by HPLC tandem mass spectrometry and plasma-total normetanephrine and total metanephrine was measured by HPLC with coulometric detection.[9,24] Results will be listed in the following sections as P-norepinephrine, P-epinephrine, P-total normetanephrine, P-free normetanephrine, P-total metanephrine, and P-free metanephrine.

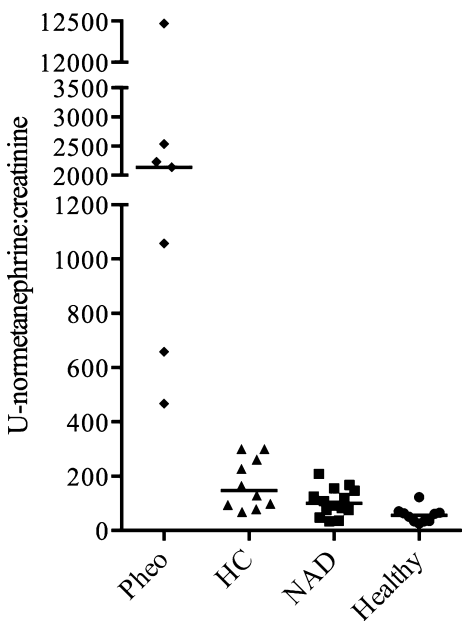

Fig. 1. Urinary normetanephrine to creatinine ratios in dogs with pheochromocytoma (PC, n = 7), dogs with hypercortisolism (HC, n = 10), dogs with nonadrenal diseases (NAD, n = 14) and in healthy dogs (n = 10). Horizontal bars represent median values.

Statistical Analysis

Data were analyzed using commercial statistical software packages.[bc] Descriptive statistics were calculated for quantitative variables by study group and analyzed for normality using the Kolmogorov-Smirnov test. Analytes with non-normal distribution in at least one group were compared using nonparametric tests. Ranges and median values are given. The dogs with PDH and dogs with AT were examined as one group (HC), because of the low number of dogs. The Kruskal–Wallis test with Dunn's post-test was used for comparison among groups (PC, HC, NAD, healthy). Correlation between free and total plasma metanephrines was analyzed by Spearman's Rank-Order Correlation. Values of $P < .05$ were considered significant.

Results

Urinary Catecholamines and Metanephrines

U-catecholamines and U-metanephrines results for dogs with PC, HC, nonadrenal diseases, and healthy dogs are depicted in Table 1. U-norepinephrine : creatinine and U-epinephrine : creatinine did not differ between dogs with PC and dogs with HC, however, U-norepinephrine : creatinine was significantly higher in dogs with PC than in dogs with NAD and healthy dogs.

U-normetanephrine : creatinine and U-metanephrine : creatinine were significantly higher in dogs with PC compared to the dogs in the other 3 groups (Fig 1). U-normetanephrine : creatinine ratios in 7 PC dogs (≥467, minimum) did not overlap with those of HC dogs (≤300, maximum), NAD dogs (≤208, maximum), or healthy dogs (≤123, maximum); however U-metanephrine : creatinine of 2 and 3 PC dogs overlapped with those of HC (≤145, maximum) and NAD (≤342, maximum) dogs, respectively. If present, statistically significant differences between dogs with HC, NAD, and healthy dogs of all urinary variables are indicated in Table 1.

Plasma Catecholamines and Metanephrines

P-catecholamines and P-metanephrines results of dogs with PC, HC, NAD and healthy dogs are depicted in Table 1. There was a marked overlap in P-epinephrine and P-norepinephrine among the results of all groups.

P-total and free normetanephrine were significantly higher in dogs with PC compared to dogs with HC, NAD, and healthy dogs. The P-total and P-free normetanephrine concentrations in only 1 of the 7 PC dogs overlapped with concentrations found in HC and NAD dogs.

P-total metanephrine was not different in dogs with PC compared to HC dogs; however, there was a significant difference compared to NAD and healthy dogs. P-free metanephrine was significantly higher in PC dogs compared to dogs of all other groups. P-total metanephrine and P-free metanephrine of 3 and 4 PC dogs overlapped with those of HC and NAD dogs, respectively. If present, statistically significant differences between dogs with HC, NAD, and healthy dogs of all plasma variables are indicated in Table 1.

Table 1. Ranges and median values of urinary catecholamines and metanephrines to creatinine ratios, plasma catecholamines and plasma-total and free metanephrines in dogs with pheochromocytoma (PC), with hypercortisolism (HC), with nonadrenal diseases (NAD), and in healthy dogs.

Parameter	PC	HC	NAD	Healthy
U-norepinephrine : creatinine	8.0–2687.0 (44.0)[a]	7.0–31.0 (14.5)[a]	2.0–26.0 (8.0)[b]	3.0–17.0 (4.5)[b]
U-epinephrine : creatinine	2.0–914.0 (13.0)	2.0–25.0 (7.5)	1.0–47.0 (6.0)	1.0–18.0 (3.0)
U-normetanephrine : creatinine	467.0–12472.0 (2137.0)[a]	68.0–300.0 (147.0)[b]	34.0–208.0 (99.5)[b]	26.0–123.0 (56.0)[c]
U-metanephrine : creatinine	134.0–4576.0 (1381.5)[a]	31.0–145.0 (56.5)[b]	28.0–343.0 (63.5)[b]	12.0–65.0 (31.5)[c]
P-norepinephrine (nmol/L)	1.1–108.0 (7.6)[a]	1.8–7.0 (2.3)[a,b]	0.6–5.1 (1.9)[b,c]	0.9–2.2 (1.6)[c]
P-epinephrine (nmol/L)	0.1–368.0 (0.7)	0.1–1.4 (0.6)	0.2–2.9 (0.8)	0.3–1.2 (0.7)
P-total normetanephrine (nmol/L)	6.5–673.0 (24.4)[a]	2.4–15.0 (6.3)[b]	2.2–7.2 (4.6)[b,c]	2.8–5.4 (3.1)[c]
P-free normetanephrine (nmol/L)	3.3–211.0 (11.8)[a]	1.5–6.6 (2.6)[b]	1.0–4.0 (2.4)[b]	0.9–-2.1 (1.4)[c]
P-total metanephrine (nmol/L)	1.6–241.0 (6.4)[a]	0.3–4.2 (2.0)[a,b]	0.5–6.3 (1.6)[b,c]	0.4–2.2 (1.4)[b,c]
P-free metanephrine (nmol/L)	1.0–102.0 (3.8)[a]	0.4–1.7 (1.2)[b]	0.5–4.1 (1.1)[b]	0.3–1.2 (0.8)[b]

Different letters indicate statistically significant differences between the groups ($P < .05$).

Comparison between Free and Total Plasma Results

There was a significant positive correlation between P-free normetanephrine and P-total normetanephrine ($r = 0.91$, $P < .001$) as well as between P-free metanephrine and P-total metanephrine ($r = 0.83$, $P < .001$).

Discussion

We were able to show that the determination of metanephrine and normetanephrine (plasma and urine) was superior in differentiating dogs with PC from dogs with HC and NAD compared to epinephrine and norepinephrine. Our results confirm earlier studies in which catecholamines and their metabolites were evaluated in dogs with PC and other diseases.[18,21] Measurement of the catecholamine metabolites in urine or plasma samples in human medicine has become the biochemical test of choice in the diagnosis of PC.[1,25] The main reason is that catecholamines are rapidly metabolized into metanephrines within the tumor, and the latter are continuously released into the circulation.[11,15,26]

In this study, U-normetanephrine : creatinine was the best variable to differentiate between PC, HC and NAD, consistent with earlier studies.[19–21] Normetanephrine was clearly superior to metanephrine. This was true for the urine as well as for the plasma test and is most likely because of the fact that canine PCs produced more norepinephrine than epinephrine. One might therefore consider limiting testing to normetanephrine. However, the number of dogs with PC in which metanephrines have been evaluated is still quite small. Although it has not been shown in dogs yet, it is possible that some PCs predominantly produce epinephrine (which is metabolized to metanephrine), as has been described in humans.[27] Those would be missed if only normetanephrine were to be measured.

HC is a differential diagnosis for PC: Both diseases might have similar clinical signs such as weakness, tachypnea, panting, polydipsia/polyuria, hypertension, as well as similar abnormalities of the adrenal glands detected via ultrasonography.[21] Because some overlap in U-normetanephrine : creatinine between PC and HC was found in our earlier study, we recommended using a cut-off value of 4 times the upper limit of normal.[21] In human medicine, it has been shown that by working with a cut-off value of 4 times the upper limit of normal the probability of a PC is nearly 100%.[1]

In this study, the calculated cut-off value of 4 times normal was somewhat higher than in our previous study (492 versus 364).[21] Using the cut-off value of the present study, one of the dogs with PC would have been missed. This means that diagnostic sensitivity would decrease if the cut-off were changed. Comparing the ages of the healthy dogs in the earlier study[21] with those of the healthy dogs in this study revealed a significant difference between the two in that the latter were significantly younger ($P = .002$; data not shown). From human medicine, it is known that free plasma normetanephrine is influenced by age and that age-adjusted cut-offs of reference intervals improve diagnostic performance.[28] The population that is used for establishing reference intervals is important. Reference interval in general, or in our case cut-off values, should best be established in a population with an age range in which the disease is expected to occur, and PCs are expected to occur predominantly in middle-aged to older dogs.

In urine, total (conjugated + free) metanephrines are measured, whereas in plasma measurement of both free and total as separate tests is available. Recently, the measurement of free metanephrines in plasma was described in veterinary medicine.[18] Determination of P-free normetanephrine was superior to P-free metanephrine to differentiate dogs with PC from dogs with HC and dogs with NAD. There was only a moderate overlap between the groups. The results presented here approximate those findings. P-free normetanephrine and P-free metanephrine were significantly higher in dogs with PC compared to the other groups and overlap was clearly less for P-free normetanephrine.

The measurement of P-total metanephrines is analytically easier than measurement of P-free metanephrines, because they are rather stable and appear in plasma in higher concentrations.[9,29,30] In humans it was shown that the sensitivity and specificity of plasma-total and plasma-free metanephrines is similar.[9] In our study, performance of P-total and P-free parameters compared

very well and the number of dogs with PC having concentrations within the range of dogs with HC was identical. Account has to be taken of the fact that the P-total metanephrines are eliminated in the urine, therefore false high values can be expected if renal function is impaired. Because P-free metanephrines are relatively independent from renal function, they are more suitable in cases of renal failure[27]; however, this issue has yet to be evaluated in dogs.

Nonadrenal illness can increase the production of catecholamines and metanephrines.[17,31–33] The impact of nonadrenal diseases can vary according to their severity. To circumvent the problem of false positive results the use of the above-mentioned cut-off values of 4 times normal is helpful. One should be aware, however, that in using this cut-off value PC might be missed because of decreased diagnostic sensitivity. If diagnostic sensitivity is high, diagnosis can be excluded based on a negative test result; on the other hand, the higher the sensitivity of a test in a biological setting, the lower its specificity, meaning more false positive results. The most important clinical task is to differentiate dogs with PC from those with HC or other pathologies that may look like PC. A cut-off point above which PC is likely even in the face of other diseases should be determined. It is worth noting that concurrent HC and PC did not have an influence on catecholamine metabolite excretion, as concentrations of the 2 dogs with both diseases did not differ from the other dogs in the PC group, and significance between the groups did not change when these 2 dogs were excluded from statistical analysis (data not shown).

The main limitation of our study is the lack of a gold standard with which the true function of the adrenal gland can be determined. We cannot exclude that we have missed more dogs with PC, as the same method was used as a 'gold standard' (cut-off of 4 times U-normetanephrine : creatinine of healthy dogs) and was at the same time being evaluated, although during the study period we did not have dogs with values in the "gray-zone" that were excluded. Obtaining histopathology on all animals is difficult and often impractical in a clinical setting.

In human medicine there is some controversy as to which test (urine or plasma) has the higher diagnostic accuracy for PC. Several authors have demonstrated a higher sensitivity of P-free metanephrines compared to U-metanephrines.[10,11,15] The specificity of the plasma test has been claimed to be lower; however, the various studies are difficult to compare because of differences in design.

In this study, U-normetanephrine : creatinine differentiated dogs with PC from dogs with HC and dogs with NAD without any overlap, whereas with regard to P-normetanephrines, there was some overlap among the groups. However, as we did find some overlap in our previous studies, this is a coincidental finding.[20,21] Moreover, it is likely that the overlap among the different groups of U-normetanephrine : creatinine ratios and also of the other parameters would have been more extensive using higher numbers of dogs.

In conclusion, determination of normetanephrine seems the best parameter to differentiate between dogs with PC and those with other diseases. So far no recommendation on whether to test urine or plasma can be made; therefore until more studies have been performed, the decision should be based on available technical facilities and the availability of dog-specific reference ranges.

Footnotes

[a] Vetoryl®, MSD Animal Health GmbH, Luzern, CH
[b] IBM SPSS, Software Packets for Macintosh, Version 19
[c] GraphPad PRISM for Macintosh, Version 6

Acknowledgments

The authors gratefully acknowledge the veterinarians of the Clinic for Small Animal Internal Medicine for their contribution of cases. We thank all of the dog owners for their assistance and willingness to take part in this study.

Conflict of interest: The authors declare that they have no potential conflicts of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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