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Reference intervals for plasma, urinary, and salivary concentrations of free metanephrines in dogs: Relevance to the diagnosis of pheochromocytoma

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Abstract

Background: Measurement of free metanephrines is recommended for screening of pheochromocytoma (PCC) but requires appropriate reference intervals (RIs).

Hypothesis/Objectives: To report RIs for plasma, urinary and salivary concentrations of free metanephrines and to determine the diagnostic performance of plasma free normetanephrine (pNMN) and metanephrine (pMN) concentrations in dogs with PCC, hypercortisolism (HC), and nonadrenal illness (NAI).

Animals: Eighty healthy dogs, 11 PCC dogs, 25 HC dogs, 6 NAI dogs.

Methods: Plasma, urine, and saliva were collected prospectively from healthy dogs, and free metanephrine concentrations were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). In addition, medical records of dogs that had plasma free metanephrine concentrations measured by LC-MS/MS between 2018-2021 were studied retrospectively.

Results: The RIs for free metanephrines in plasma, urine and saliva are reported. Dogs with PCC had significantly higher pNMN than dogs with HC ($P < .001$) and NAI ($P = .002$). The PCC dogs had significantly higher pMN than HC dogs ($P < .001$), but not higher than NAI dogs ($P = .29$). Using the upper reference limit, pNMN (>3.56 nmol/L) showed high sensitivity (100%, 95% confidence interval [CI]: 72-100)

Abbreviations: 3MT, 3-methoxytyramine; ADH, adrenal-dependent hypercortisolism; ASVCP, American Society for Veterinary Clinical Pathology; CI, confidence interval; CT, computed tomography; HC, hypercortisolism; HPLC-ED, high-pressure liquid chromatography with electrochemical detection; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LRL, lower reference limit; MN, metanephrine; NAI, nonadrenal illness; NMN, normetanephrine; p3MT, plasma free 3-methoxytyramine; PCC, pheochromocytoma; PDH, pituitary-dependent hypercortisolism; pMN, plasma free metanephrine; pNMN, plasma free normetanephrine; RI, reference interval; ROC, receiver operating characteristic; s3MT, salivary free 3-methoxytyramine; sMN, salivary free metanephrine; sNMN, salivary free normetanephrine; u3MT, urinary free 3-methoxytyramine; UCCR, urinary corticoid: creatinine ratio; uCr, urinary creatinine; uMN, urinary free metanephrine; uNMN, urinary free normetanephrine; URL, upper reference limit; WCI/WRI, ratio of the width of the 90% confidence interval: width of the reference interval.

and specificity (94%, 95% CI: 79-99) for diagnosis of PCC, whereas pMN (>2.49 nmol/L) showed moderate sensitivity (73%, 95% CI: 39-94) and high specificity (94%, 95% CI: 79-99).

Conclusions and Clinical Importance: With establishment of these RIs, biochemical testing for PCC in dogs can be substantially improved. Measurement of pNMN is superior to pMN in dogs with PCC.

KEYWORDS
adrenal tumor, canine, catecholamines, normetanephrine

1 | INTRODUCTION

Pheochromocytomas (PCCs) are neuroendocrine tumors arising from chromaffin cells of the adrenal medulla. When biochemically functional, they secrete excessive amounts of catecholamines, causing a variety of nonspecific, potentially fatal, clinical signs, such as tachyarrhythmias, hypertension, weakness, tachypnea, abdominal pain, and polyuria and polydipsia.[1-3] The diagnosis of PCC is based on clinical presentation, diagnostic imaging findings, and biochemical detection of catecholamines (epinephrine and norepinephrine) and their metabolites, metanephrine (MN) and normetanephrine (NMN).[3,4] Measurement of metanephrines has higher diagnostic accuracy because of continuous intratumoral production and secretion of metanephrines compared to episodic secretion of catecholamines.[5] In humans, biochemical testing for PCC should include measurements of plasma free metanephrines or urinary free metanephrines.[6] Recently, measurement of salivary metanephrines has been shown to be a promising tool in the biochemical diagnosis of PCC in humans.[7] Additionally, measurement of 3-methoxytyramine (3MT), a metabolite of dopamine, is useful for diagnosis of dopamine-producing PCCs.[8]

In dogs, routine biochemical testing for PCC only started 10-15 years ago and consists of evaluation of plasma and urinary catecholamines and metanephrines, either by high-pressure liquid chromatography with electrochemical detection (HPLC-ED)[9-13] or by liquid chromatography-tandem mass spectrometry (LC-MS/MS).[13-15] Measurement of metanephrines has a better diagnostic performance than measurement of catecholamines.[11,14] Currently, there is no consensus on whether to use urine or plasma for determination of metanephrine concentrations in dogs.[13] Despite several studies reporting on these biomarkers in healthy dogs, dogs with PCC, dogs with hypercortisolism (HC), and dogs with nonadrenal illness (NAI),[9-15] a reference interval (RI) in healthy dogs has not been determined for any of these biomarkers, complicating diagnostic screening for PCC.

Our aim was to report RIs for plasma, urinary and salivary free metanephrines and 3MT in healthy dogs measured by LC/MS-MS, and to explore the diagnostic performance of plasma free normetanephrine (pNMN) and plasma free metanephrine (pMN) in patients with histologically-confirmed PCC.

2 | MATERIALS AND METHODS

2.1 | Reference intervals for plasma, urinary and salivary free metanephrines and 3MT

Healthy control dogs of various breeds, age and body weight owned by veterinary students and staff of Utrecht University's Department of Clinical Sciences, Faculty of Veterinary Medicine were enrolled between September 2016 and January 2018. Informed written consent was obtained from dog owners before study participation. All dogs were considered healthy based on unremarkable history and routine physical examination. Dogs were excluded if they had been treated with glucocorticoids, antibiotics or any medication possibly influencing concentrations of metanephrines (e.g., alpha-adrenoreceptor antagonists, metoclopramide[16]) within the last 3 weeks.

2.2 | Diagnostic performance plasma free metanephrines

Medical records of dogs that had free metanephrines measured in plasma (pNMN and pMN) between January 2018 and October 2021 were reviewed. Dogs with PCC, HC, and NAI were included. In these patients, no measurements of urinary or salivary free metanephrines were performed. Similarly, 3MT was not measured in these patients. The diagnosis of PCC was confirmed by histopathology and immunohistochemistry using the adrenomedullary markers synaptophysin and chromogranin A.[17] The diagnosis of HC was confirmed in dogs highly suspected to have the disease (in light of compatible history, physical examination findings and laboratory abnormalities) by a lack of adequate cortisol suppression in an IV low-dose dexamethasone suppression test (IV-LDDST) or by increased urinary corticoid: creatinine ratios (UCCRs; $>8.3 \times 10^{-6}$).[18-20] Discrimination between pituitary-dependent hypercortisolism (PDH) and adrenal-dependent hypercortisolism (ADH) was done by IV-LDDST and UCCR in combination with a high-dose dexamethasone suppression test,[21] measurement of endogenous ACTH (Immulite 2000, Siemens Medical Solutions Diagnostics, Los Angeles, USA) and diagnostic imaging of the adrenal and pituitary glands. Dogs with NAI only were included if PCC was considered as a differential diagnosis, and if complete diagnostic evaluation

was performed, including physical examination, CBC, serum biochemistry, and diagnostic imaging of the adrenal glands. Further diagnostic testing varied based on the presenting complaints (e.g., thoracic radiographs, computed tomography [CT], echocardiography, electrocardiography, urinalysis, fecal examination). Dogs with NAI were excluded if adrenal imaging was not performed or if they were diagnosed with an adrenal mass on diagnostic imaging.

2.3 | Sample collection

All healthy control dogs underwent at least a 12 hour fast before samples were collected. Voided morning urine samples were collected at home on the day that dogs were presented to the Department of Clinical Sciences. Samples were kept refrigerated at home and were transported in a cooling box with ice packs. Upon arrival (T_0), blood was collected from each dog by jugular venipuncture. After collection, blood was transferred into EDTA tubes that were kept on ice until further processing. Next, saliva was collected by swabbing the buccal cavity of the dogs using a pediatric dental rope for approximately 2 minutes. The rope was transferred to a Salivette (Sarstedt, Nümbrecht, Germany). In a subset of the healthy control dogs (n = 37), a second blood sample was collected (T_1) to assess whether the stay at the hospital influenced plasma free metanephrines and 3MT concentrations. Within 15 minutes after blood collection, EDTA tubes were centrifuged in a refrigerated centrifuge (3500 rpm, 12 minutes) and plasma was separated. The Salivette was centrifuged (4000 rpm, 2 minutes) to yield the saliva sample. Directly after processing, all samples were stored at −20°C (for a maximum of 1 month) until they were transported on ice to the Department of Laboratory Medicine at the University Medical Center Groningen. At the Department of Laboratory Medicine, samples were stored at −80°C until analysis (within 6 months of sample collection).

In patients with PCC, HC, and NAI, blood was collected after at least a 12 hour fast. After collection, blood was transferred into EDTA tubes that were kept on ice. Within 15 minutes after blood collection, EDTA tubes were centrifuged in a refrigerated centrifuge (3500 rpm, 12 minutes) and plasma was separated. Plasma samples were stored at −20°C (for a maximum of 3 days) until they were transported on ice to Euregio Laboratory Services. At Euregio Laboratory Services, samples were stored at −80°C until analysis (within 1 week of sample collection).

2.4 | Measurement of free metanephrines and 3MT

Measurement of metanephrines and 3MT in plasma, urine and saliva consisted of quantification of free (unconjugated) fractions. At the Department of Laboratory Medicine, concentrations of plasma free and salivary free metanephrines and 3MT were analyzed by an automated LC-MS/MS method using direct-matrix derivatization, as previously described and validated for samples from humans.[7,22] Urinary free metanephrines and 3MT were analyzed using essentially the same method as for plasma free metanephrines, but fully validated for human urine. Interassay imprecision was determined at n = 20 different days at 3 different concentrations. Interassay imprecision was below 3.3%, 5.1%, and 3.5% for urinary NMN, MN, and 3MT, respectively. Concentrations of urinary free MN, NMN, and 3MT (uMN, uNMN, u3MT) were normalized to urinary creatinine (uCr) concentrations and expressed as ratios, as reported previously.[10,11] Concentrations of plasma free NMN and MN in dogs with PCC, HC, and NAI were analyzed by LC-MS/MS at Euregio Laboratory Services.

2.5 | Statistical analysis

Determination of the 95% RIs was conducted according to the American Society for Veterinary Clinical Pathology (ASVCP) 2012 guidelines,[23] using MedCalc for Windows, version 20.011 (MedCalc Software, Ostend, Belgium). Data were assessed for normality of distribution using the Shapiro-Wilk test. Because data for plasma, urinary and salivary free metanephrines and 3MT were not normally distributed, log or Box-Cox transformations were performed. Robust methods were used to determine RIs. Bootstrap methods were used to determine 90% confidence intervals (CIs) around reference limits. The Dixon/Reed method was used to identify outliers.

Further data analysis was performed using the statistical software program IBM SPSS Statistics for Windows, version 27 (IBM Corp., Armonk, NY). A nonparametric correlation analysis (Spearman's rank correlation coefficient) was used to calculate the correlation between age and metanephrines or 3MT, body weight and metanephrines or 3MT, and tumor size and plasma free metanephrines. Differences in results between 2 groups were assessed using the nonparametric Mann-Whitney *U*-test. To compare results among multiple groups, the Kruskal-Wallis test was used. When this test indicated significance, differences between individual groups were assessed by post hoc analysis, and *P* values were adjusted by the Bonferroni correction method to adjust for multiple comparisons. Values at T_0 and T_1 were compared using the nonparametric Wilcoxon signed-rank test for paired samples. Values were considered statistically significant at $P < .05$. Using the upper reference limit (URL) as a cutoff, the sensitivity and specificity of pNMN and pMN were calculated. The 95% CIs around sensitivity and specificity estimates were based on the binomial distribution. Based on receiver operating characteristic (ROC) curve data, Youden's index was used to determine optimal cutoff points for pNMN and pMN. Receiver operating characteristic curves were generated to compare the diagnostic performance of pNMN and pMN for distinguishing dogs with PCC from dogs with HC and NAI.

3 | RESULTS

3.1 | Study population

For determination of RIs for plasma, urinary and salivary free metanephrines and 3MT, a reference population of 80 healthy dogs was used.

For determination of the diagnostic performance of plasma free metanephrines, 11 dogs with PCC, 25 dogs with HC and 6 dogs with NAI were included. The characteristics (breeds, sex, age and body weight) of the study population can be found in Supplementary file 1.

All HC dogs had diagnostic imaging of the adrenal glands, pituitary gland, or both, with CT of the pituitary gland and abdomen in 21 dogs, abdominal CT in 1 dog, abdominal ultrasound examination in 2 dogs, and magnetic resonance imaging of the pituitary gland and abdominal ultrasound examination in 1 dog. In 23 dogs, UCCRs were performed (mean, 54.2 $\times$ 10^{-6}; median, 33.2 $\times$ 10^{-6}; first quartile, 19.5 $\times$ 10^{-6}; third quartile, 70.4 $\times$ 10^{-6}; range, 7.4-199 $\times$ 10^{-6}), whereas an IV-LDDST was performed in 3 dogs and endogenous ACTH was measured in 23 dogs. All dogs had UCCRs above the decision threshold (>8.3 $\times$ 10^{-6}) except for 1 dog, in which the UCCR (7.4 $\times$ 10^{-6}) was somewhat below the decision threshold. In this dog, the diagnosis of PDH was based on compatible history, physical examination findings and laboratory abnormalities, the presence of a pituitary mass, and good response to trilostane. Ten dogs were diagnosed with ADH. Three of 10 dogs with ADH underwent adrenalectomy, and histopathology of the adrenal glands confirmed the presence of adrenocortical tumors in these dogs. Five dogs were diagnosed with PDH, and 4 additional dogs were diagnosed with PDH but also had adrenal masses (unilateral mass in 3 dogs, bilateral masses in 1 dog). Histopathology of the adrenal glands was available in 2 of these 4 dogs and showed the presence of an adrenocortical carcinoma in 1 dog and the presence of both an adrenocortical carcinoma and adenoma in 1 dog. In the remaining 6 dogs, discrimination between ADH and PDH was not possible based on endocrine testing and diagnostic imaging. All 6 dogs had both pituitary and adrenal masses (unilateral adrenal mass in 2 dogs, bilateral adrenal masses in 4 dogs). Histopathology was available in 2 of 6 dogs and showed the presence of cortical adenomas.

For the NAI dogs, presenting complaints and diagnoses were as follows: 1 dog had episodic tremors (no underlying cause found), 1 dog presented for evaluation of hypertension and chronic diarrhea (diagnosed with primary hypertension and food-responsive enteropathy), 1 dog presented with panting, restlessness, flatulence, tremors, and abdominal pain (diagnosed with tracheal collapse, bronchomalacia, and food-response enteropathy), 1 dog showed exercise intolerance, panting, tremors, and decreased activity (diagnosed with osteoarthritis and aortic stenosis), 1 dog presented with progressive panting, exercise intolerance, weight gain, stridor, flatulence, lameness, and coat changes (diagnosed with hypothyroidism and osteoarthritis), and the remaining dog presented because of collapse, exercise intolerance, lethargy, polyuria and polydipsia, and hyporexia and was lost to follow-up before a final diagnosis could be obtained. Four dogs received no medication in the 4 weeks before plasma free metanephrine measurement. The dog presented for evaluation of hypertension was receiving amlodipine (0.26 mg/kg PO q24h), whereas the dog with hypothyroidism and osteoarthritis was receiving levothyroxine (12 µg/kg PO q12h), tramadol (3 mg/kg PO q8h) and firocoxib (7.5 mg/kg PO q24h).

Healthy dogs were significantly younger than dogs with PCC (P < .001), HC (P < .001), and NAI (P = .02). No significant differences in body weight were found among the reference population, dogs with PCC, dogs with HC, and dogs with NAI.

3.2 | Reference intervals for plasma free metanephrines and 3MT

The reference population of 80 healthy control dogs was used to determine RIs for pNMN, pMN, and plasma free 3MT (p3MT). The 95% RIs with 90% CI for the lower and upper limits are presented in Table 1.

3.3 | Reference intervals for urinary free metanephrines and 3MT

Owners were unable to obtain voided morning urinary samples in 6 dogs. Unfortunately, results for urinary creatinine were lost from our database for 37 dogs. Therefore, the remaining 37 healthy dogs of the reference population were used to determine RIs for urinary free metanephrines and 3MT. The 95% RI with 90% CI for the lower and upper limits, and the median and range for urinary free NMN, MN, and 3MT (normalized to urinary creatinine concentration) are presented in Tables 1 and 2. Distribution of uNMN/uCr, uMN/uCr, and u3MT/uCr is represented in Figure 1.

3.4 | Reference intervals for salivary free metanephrines and 3MT

The reference population of 80 healthy dogs also was used to determine RIs for salivary free metanephrines and 3MT. In 4 samples, salivary free NMN (sNMN) could not be determined, resulting in a final number of 76 reference individuals for the sNMN RI. Normality for salivary free 3MT (s3MT) could not be established despite several transformation trials. The 95% RIs with 90% CI for the lower and upper limits are presented in Table 1.

3.5 | Reference population: influence of sex, age, body weight, and stay at the hospital

Plasma, urinary and salivary free concentrations of MN, NMN and 3MT did not differ between male and female dogs. A weak, positive correlation was found between age and pNMN (rho = 0.26, P = .02), age and sNMN (rho = 0.35, P = .002), and age and sMN (rho = 0.32, P = .003). A weak, negative correlation was found between body weight and pNMN (rho = −0.24, P = .03), body weight and sNMN (rho = −0.27, P = .02), and body weight and sMN (rho = −0.32, P = .003).

TABLE 1 95% RIs with 90% CI for lower and upper limits for plasma, urinary and salivary free metanephrines and 3MT in healthy dogs

	n	95% RI	90% CI LRL	90% CI URL	Method	Distribution (transformation)	*P*-value normality test
pNMN (nmol/L)	80	0.90-3.56	0.81-1.00	3.16-4.02	Robust[a]	Gaussian (log)	.16
pMN (nmol/L)	80	0.35-2.49	0.30-0.41	2.09-2.97	Robust[a]	Gaussian (log)	.13
p3MT (nmol/L)	80	0.16-1.31	0.14-0.20	1.11-1.60	Robust[a]	Gaussian (log)	.90
uNMN/uCr (nmol/mmol)	37	16.8-97.4	14.0-20.6	78.6-120	Robust[a]	Gaussian (log)	.89
uMN/uCr (nmol/mmol)	37	7.96-65.6	6.16-10.4	50.1-83.0	Robust[a]	Gaussian (log)	.79
u3MT/uCr (nmol/mmol)	37	27.9-136	23.8-33.5	111-162	Robust[a]	Gaussian (log)	.88
sNMN (nmol/L)	76	0.04-7.99	0.0002-0.16	6.67-9.26	Robust[a]	Gaussian (Box-cox)	.24
sMN (nmol/L)	80	0.03-2.95	0.01-0.07	2.47-3.47	Robust[a]	Gaussian (Box-cox)	.11
s3MT (nmol/L)	80	0.007-2.54	0.001-0.02	2.07-3.10	Robust	Non-Gaussian	<.05

Note: A threshold of $P < .05$ of the Shapiro-Wilk normality test was used to determine if the distribution was Gaussian or non-Gaussian (after transformation).
Abbreviations: CI, confidence interval; LRL, lower reference limit; n, final number of reference individuals; p3MT, plasma free 3-methoxytyramine; pMN, plasma free metanephrine; pNMN, plasma free normetanephrine; RI, reference interval; s3MT, salivary free 3-methoxytyramine; sMN, salivary free metanephrine; sNMN, salivary free normetanephrine; u3MT/uCr, urinary free 3-methoxytyramine to creatinine ratio; uMN/uCr, urinary free metanephrine to creatinine ratio; uNMN/uCr, urinary free normetanephrine to creatinine ratio; URL, upper reference limit.
[a]Data were transformed (using log or Box-cox transformation) to Gaussian distribution before applying the robust method.

TABLE 2 Concentrations of plasma, urinary and salivary free metanephrines and 3MT in healthy dogs, and of plasma free metanephrines in 11 dogs with pheochromocytoma (PCC), 25 dogs with hypercortisolism (HC), and 6 dogs with nonadrenal illness (NAI)

Analyte	95% RI	Healthy dogs	PCC dogs	HC dogs	NAI dogs
pNMN (nmol/L)	0.90-3.56	1.76 (0.83-4.34)	5.9 (3.7-24.8)	2.2 (<0.5-3.8)	2.3 (1.4-2.8)
pMN (nmol/L)	0.35-2.49	0.93 (0.41-3.70)	3.7 (1.2-7.5)	0.7 (<0.3-2.0)	1.4 (0.7-3.2)
p3MT (nmol/L)	0.16-1.31	0.46 (0.10-2.11)	N/A	N/A	N/A
uNMN/uCr (nmol/mmol)	16.8-97.4	41.8 (16.7-108)	N/A	N/A	N/A
uMN/uCr (nmol/mmol)	7.96-65.6	23.1 (5.5-66.3)	N/A	N/A	N/A
u3MT/uCr (nmol/mmol)	27.9-136	58.8 (28.5-133)	N/A	N/A	N/A
sNMN (nmol/L)	0.04-7.99	2.09 (<0.01-10.9)	N/A	N/A	N/A
sMN (nmol/L)	0.03-2.95	0.70 (<0.04-4.28)	N/A	N/A	N/A
s3MT (nmol/L)	0.007-2.54	0.46 (<0.02-3.62)	N/A	N/A	N/A

Note: Values are expressed as median (range).
Abbreviations: HC, hypercortisolism; N/A, not applicable; NAI, nonadrenal illness; p3MT, plasma free 3-methoxytyramine; PCC, pheochromocytoma; pMN, plasma free metanephrine; pNMN, plasma free normetanephrine; RI, reference interval; s3MT, salivary free 3-methoxytyramine; sMN, salivary free metanephrine; sNMN, salivary free normetanephrine; u3MT/uCr, urinary free 3-methoxytyramine to creatinine ratio; uMN/uCr, urinary free metanephrine to creatinine ratio; uNMN/uCr, urinary free normetanephrine to creatinine ratio.

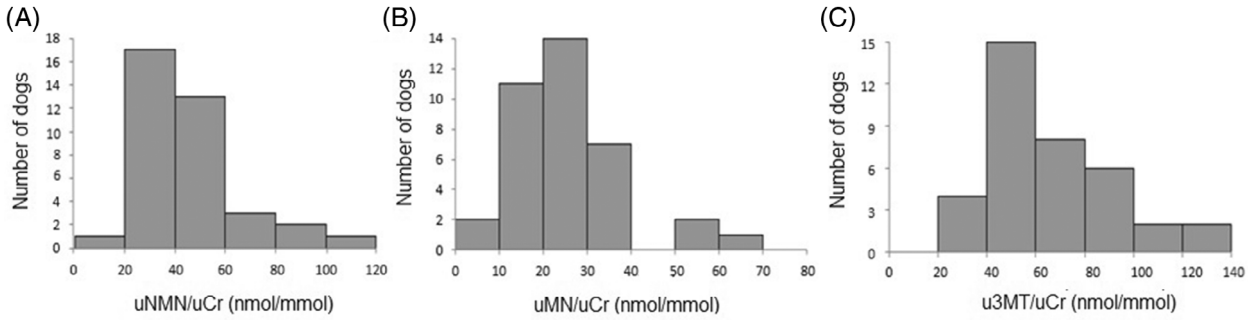

FIGURE 1 Distribution of uNMN/uCr, uMN/uCr, and u3MT/uCr in 37 healthy adult dogs. The y-axis corresponds with the number of dogs having analyte concentrations reported on the x-axis. uNMN/uCr, urinary normetanephrine to creatinine ratio; uMN/uCr, urinary metanephrine to creatinine ratio; u3MT/uCr, urinary 3-methoxytyramine to creatinine ratio

The time between collection of the first blood sample (T_0), which occurred directly upon arrival, and collection of the second blood sample (T_1), which occurred after a physical examination and collection of saliva, was 20-30 minutes. Plasma free NMN ($P = .01$), pMN ($P = .04$) and p3MT ($P = .01$) values were significantly higher at T_1 than at T_0 (Table 3, Figure 2).

TABLE 3 Results for pNMN, pMN, and p3MT in 37 healthy control dogs directly upon arrival (T_0) and after the visit at the hospital (T_1)

Analyte	T_0	T_1
pNMN (nmol/L)	1.6 (1.1-4.3)	2.0 (1.0-3.9)[a]
pMN (nmol/L)	0.96 (0.42-3.7)	1.03 (0.42-3.8)[a]
p3MT (nmol/L)	0.52 (0.10-1.1)	0.56 (0.09-1.4)[a]

Note: Values are expressed as median (range). pNMN, plasma normetanephrine; pMN, plasma metanephrine; p3MT, plasma 3-methoxytyramine.
*Statistically significant difference ($P < .05$) between T_0 and T_1.

3.6 | Diagnostic performance of plasma free metanephrines

Dogs with PCC had significantly higher pNMN than dogs with HC ($P < .001$) and NAI ($P = .002$). The PCC dogs had significantly higher pMN than HC dogs ($P < .001$), but not than NAI dogs ($P = .29$; Table 2, Figure 3).

Plasma free NMN concentration was above the RI for all PCC dogs, whereas pMN concentration was within the RI for 3 of 11 PCC dogs. In 2 of 25 HC dogs, pNMN concentration was just above the RI, whereas pMN concentration was within the RI for all dogs. For all NAI

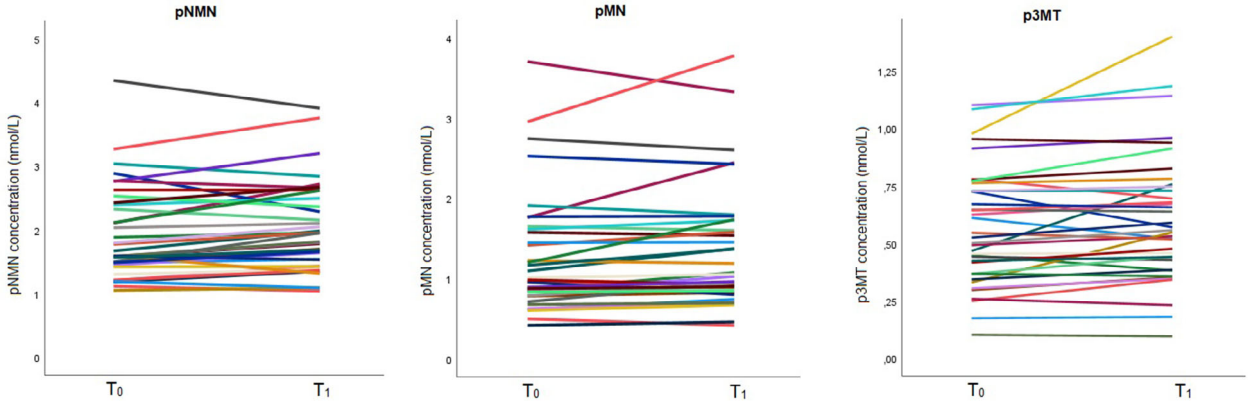

FIGURE 2 Plasma NMN, MN, and 3MT directly upon arrival (T_0) and after the visit at the hospital (T_1) in 37 healthy dogs. pNMN, plasma normetanephrine; pMN, plasma metanephrine; p3MT, plasma 3-methoxytyramine

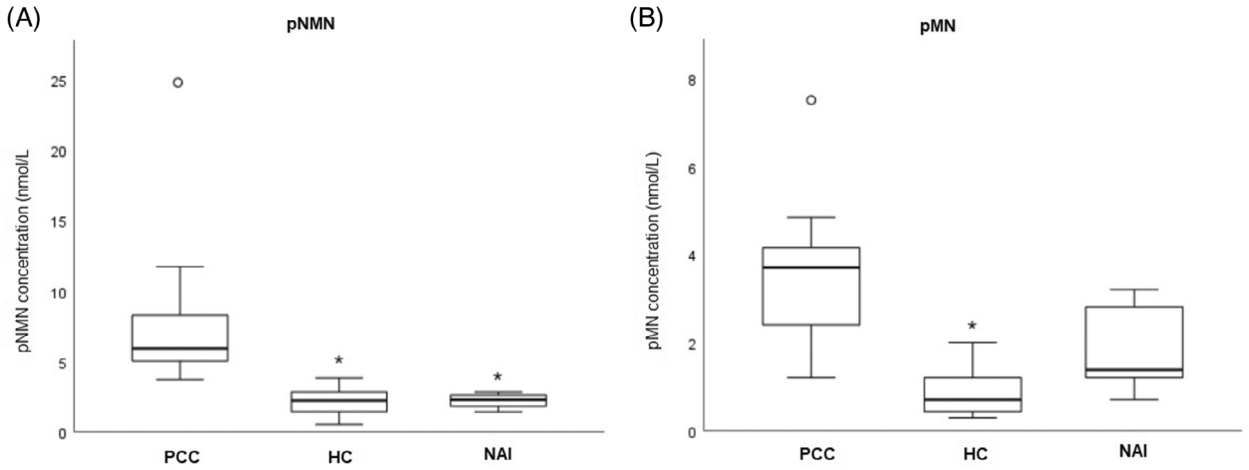

FIGURE 3 Comparison of (A) plasma normetanephrine (pNMN) concentrations and (B) plasma metanephrine (pMN) concentrations between dogs with pheochromocytoma (PCC) (n = 11), hypercortisolism (HC) (n = 25), and nonadrenal illness (NAI) (n = 6). The box represents the inter quartile range (ie, the second and third quartiles). The line within the box represents the median. The whiskers represent the range, extending to a maximum of 1.5-times the interquartile range. Outliers are represented by circles above the whiskers. *Statistically significant difference ($P < .05$) compared to dogs with PCC

TABLE 4 Sensitivity, specificity, and Youden's index for the diagnosis of pheochromocytoma of the upper reference limit of pNMN and pMN, and of different cutoff points for pNMN and pMN based on ROC curve data

Cutoff points	Sensitivity (%)	95% CI (%)	Specificity (%)	95% CI (%)	Youden's index
URL pNMN (3.56 nmol/L)	100	72-100	94	79-99	.94
pNMN >2.9 nmol/L	100	72-100	87	70-96	.87
pNMN >3.1 nmol/L	100	72-100	94	79-99	.94[a]
pNMN >3.7 nmol/L	91	59-100	97	83-100	.88
pNMN >3.8 nmol/L	82	48-98	100	89-100	.82
URL pMN (2.49 nmol/L)	73	39-94	94	79-99	.67
pMN >1.5 nmol/L	82	48-98	84	66-95	.66
pMN >1.6 nmol/L	82	48-98	90	74-98	.72[a]
pMN >2.0 nmol/L	73	39-94	94	79-99	.67
pMN >2.8 nmol/L	64	31-89	97	83-100	.61

Abbreviations: CI, confidence interval; pMN, plasma free metanephrine; pNMN, plasma free normetanephrine; ROC, receiver operating characteristic; URL, upper reference limit.
[a]Optimal cutoff point for pNMN and pMN based on the highest Youden's index.

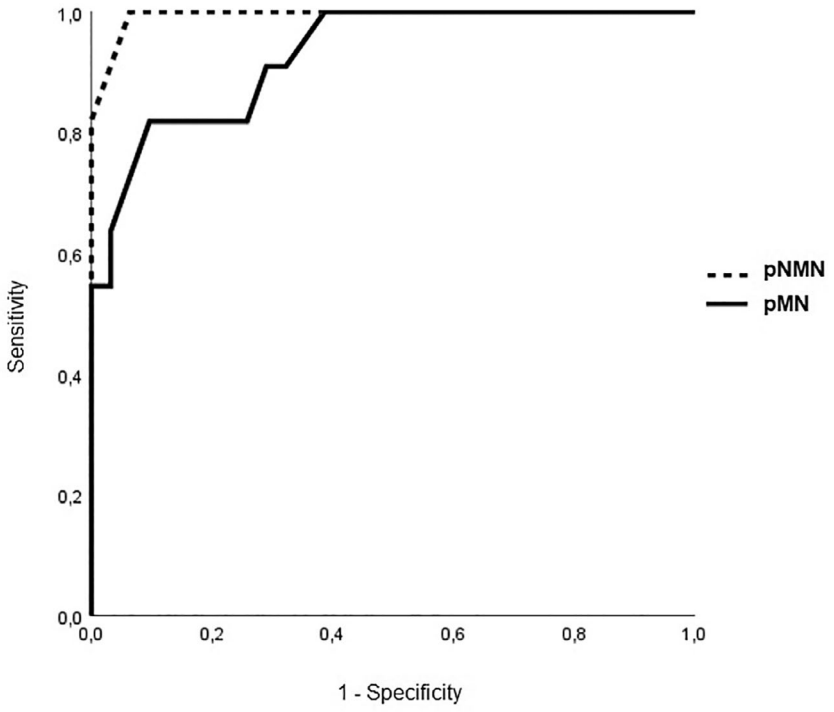

FIGURE 4 ROC curves to compare the performance of the discriminatory value of pNMN concentration (dashed line) and pMN concentration (solid line) for distinguishing dogs with PCC from dogs with HC and NAI

dogs, pNMN concentration was within the RI, whereas pMN concentration was above the RI in 2 of 6 dogs.

The sensitivity, specificity and Youden's index of the URL of pNMN and pMN are presented in Table 4. Combining pNMN and pMN (ie, PCC is considered present when both tests are positive) resulted in a sensitivity of 73% (95% CI, 39-94) and specificity of 100% (95% CI, 72-100). Different cutoffs for pNMN and pMN, with their corresponding sensitivities, specificities and Youden's indices, are presented in Table 4. The optimal cutoff for pNMN was >3.1 nmol/L, and >1.6 nmol/L for pMN. Based on ROC curves for pNMN and pMN, both showed excellent discrimination for the diagnosis of PCC, with an area under the curve of 0.99 for pNMN (95% CI, 0.98-1.0), and of 0.93 (95% CI, 0.85-1.0) for pMN (Figure 4).

3.7 | Correlation tumor size and plasma free metanephrines

A moderate positive correlation was found between tumor size and pMN (rho = 0.62, $P = .03$), but no significant correlation was found between tumor size and pNMN ($P = .18$).

4 | DISCUSSION

Ours is the first study to report 95% RIs for plasma, urinary and salivary free NM, NMN, and 3MT in a large population of healthy dogs. Plasma free NMN showed excellent diagnostic performance, with high sensitivity and specificity for the diagnosis of PCC when using the URL for pNMN (3.56 nmol/L), whereas pMN showed moderate sensitivity and high specificity for the diagnosis of PCC when using the URL (2.49 nmol/L). The optimal cutoff for pNMN (>3.1 nmol/L) gave identical sensitivity, specificity and Youden's index, whereas the optimal cutoff for pMN (>1.6 nmol/L) gave an increased sensitivity but slightly decreased specificity compared to the URL, with a slightly improved Youden's index. Combining both tests did not improve the diagnostic yield beyond that of solely using pNMN, because specificity was improved only slightly, and sensitivity worsened considerably. With these RIs, the reliability of biochemical testing for PCC in dogs can be substantially improved.

In our study, the reported 90% CIs demonstrate the uncertainty in the lower reference limits (LRLs) and URLs. Although ideally the ratio of the width of the 90% CI : width of the RI (WCI/WRI) should be <0.2,[23] the median WCI/WRI of the URL was 0.41 (range, 0.32-0.57), whereas the median WCI/WRI of the LRL was 0.05 (range, 0.008-0.09; see Supplementary file 2). The imprecision of the URL can be explained by the rather small numbers of reference individuals, especially for the population used for urinary free metanephrine RIs, and right-skewed distributions. In response to the ASVCP 2012 guidelines, it was reported that the recommendation of a WCI/WRI <0.2 may be challenged, because in log-Gaussian distributions, the WCI/WRI of the URL was >0.2 in all cases with n < 120.[24] This issue of imprecision emphasizes the fact that the diagnosis of PCC should be based on clinical presentation, diagnostic imaging findings and endocrine testing, and not only on increased metanephrine concentrations.

In dogs, measurement of NMN for the diagnosis of PCC is superior to measurement of MN, both in plasma and urine.[10,11,13-15] Our findings show that pNMN had better diagnostic performance than pMN to differentiate PCC from HC and NAI, which is consistent with earlier studies. The sensitivity and specificity for pNMN and pMN in our study are comparable to previous reported results.[14] However, the URLs for pNMN (3.56 nmol/L) and pMN (2.49 nmol/L) in our study are considerably lower than in a previous study, where URLs of 5.52 nmol/L and 4.18 nmol/L were used as cutoffs for pNMN and pMN, respectively.[14] If these cutoffs would have been used in our study, sensitivity of pNMN and pMN would have decreased markedly (to 55% and 27%, respectively), meaning that PCC would have been missed in 45% to 73% of cases. Moreover, using a higher cutoff would have increased specificity only minimally (to 100%, compared to 94%, in our study), emphasizing the drawbacks of using these high cutoffs. Plasma NMN concentrations in PCC dogs were markedly lower in our study than in previous studies,[13,14] whereas pMN concentrations were moderately lower to comparable. Similar to our study, LC-MS/MS was used for free metanephrine measurement in both previous studies, which demonstrates good agreement among different laboratories.[25] The difference in metanephrine concentrations might be explained by the fact that, today, PCCs are diagnosed earlier in the course of the disease because of increasing awareness of this disease among clinicians and increasing use of diagnostic imaging. Despite the high specificity of pNMN and pMN in our study, the use of lower cutoffs might increase the risk of false-positive results, which should be taken into account when screening for PCC.

In our study, measurement of free fractions of metanephrines and 3MT in plasma, urine and saliva was performed, which offers advantages to measurement of total (free + conjugated) metanephrines and also offers better diagnostic accuracy.[26,27] This result is in contrast with most earlier studies in dogs, which evaluated the diagnostic performance of urinary total metanephrines.[10,11,13,28] When comparing results of urinary metanephrines among different studies, this factor has to be taken into account. In addition, differences in the analytic methods used (HPLC-ED versus LC-MS/MS) must be taken into consideration. Today, LC-MS/MS is the method of choice for low-cost, high-throughput measurements of metanephrines with high detection sensitivity and specificity in humans.[29] Accordingly, LC-MS/MS was used in our study. A limitation of our study is that plasma free metanephrines of dogs with PCC, HC and NAI were measured by a different laboratory than the laboratory that was used for the reference population. However, both laboratories used LC-MS/MS for metanephrine measurements, which has been shown to provide comparable results among laboratories.[25]

Traditionally, acidification of the urine was needed for catecholamine determination, and it was assumed that acidification also was necessary for metanephrine determination.[28] Acidification is cumbersome and may interfere with measurement of other common analytes in the urine. Studies in humans and a recent study in dogs showed that acidification is not necessary for adequate measurement of urinary metanephrines.[15,30,31] Moreover, acidification using hydrochloric acid can result in deconjugation of sulfate-conjugated metabolites, which can lead to spuriously high measured concentrations of free metabolites.[32] Therefore, urinary acidification was not performed in our study.

In humans, initial biochemical testing for PCCs should include measurement of plasma free metanephrines or urinary free metanephrines.[6] More recent studies suggest that plasma measurements have superior diagnostic accuracy compared to urinary measurements, at least in a subset of patients with PCC.[33] Currently, there is no consensus on whether to use urine or plasma for metanephrine determination in dogs.[13] Because only plasma metanephrines, and not urinary metanephrines, were measured in our population of dogs with PCC, HC, and NAI, we cannot draw any conclusions regarding the diagnostic performance of the reported RIs of urinary free metanephrines.

In humans, measurement of p3MT concentrations as an additional component of the standard panel of plasma metanephrines is used to identify PCCs that produce only dopamine. However, because these tumors are rare, detection of PCCs is improved only modestly.[8] It is unknown whether dogs have PCCs that are exclusively dopamine-producing. Therefore, it would be relevant to determine 3MT in dogs with PCCs. Furthermore, such an investigation would be

of interest because p3MT may serve as a biomarker of malignancy in humans with PCC.[34]

In humans, metanephrines can be readily measured in saliva of healthy subjects.[35] Recently, it was shown that the diagnostic accuracy of salivary metanephrines was 88%, which makes it a promising tool in the diagnosis of PCC.[7] Theoretically, the use of saliva offers advantages over plasma and urine because samples can be collected easily and noninvasively at home, and collection is minimally stressful. Although we were able to detect salivary free metanephrines and 3MT using LC-MS/MS in healthy dogs, we found it difficult to collect sufficient saliva using the dental rope, especially if experience with this collection method is limited. Consequently, this concern limits the potential advantages of using saliva for determination of metanephrines in dogs, especially if performed by owners at home. Nevertheless, it would be interesting to measure sNMN, sMN, and s3MT in dogs with PCC, HC, and NAI.

None of the healthy dogs in our study were treated with metoclopramide, alpha-adrenoreceptor antagonists, or other medications known to affect metanephrine concentrations.[16] Phenoxybenzamine is an important cause for false-positive results in the screening for PCC, because it can increase concentrations of NMN.[36] None of the dogs with PCC received phenoxybenzamine before testing. Although we attempted to include only healthy animals based on unremarkable history and physical examination, the health screening did not include CBC, serum biochemistry, or urinalysis. Therefore, some dogs may have had subclinical disease. In addition, because some dogs with PCC are asymptomatic,[1,2] it would have been preferable to perform abdominal ultrasound examination in all dogs to exclude the presence of an adrenal mass.

In human medicine, a positive relationship has been found between advancing age and increased pNMN. Age-adjusted cutoffs of RIs for pNMN are used because they improve diagnostic test performance.[37] In our study, a positive, albeit weak, correlation between age and pNMN was found. Ideally, the demographics of the reference population should mimic the population for which the RI will be used.[23] In our study, healthy dogs were significantly younger than PCC dogs. Although the median age was comparable to previous studies that evaluated plasma metanephrines in healthy dogs,[13,14] the upper age range was considerably higher in our study (14 years compared to 7 years; and 27% of dogs were >7 years). However, considering the weak positive correlation between age and pNMN, inclusion of older animals in the reference population would be preferable. Because higher age also was associated with increased sNMN and sMN concentrations in our study, age also must be taken into consideration when interpreting sNMN and sMN in dogs.

Previous studies evaluated the influence of stress on urinary metanephrine excretion in healthy dogs.[9,10] Stress associated with a hospital visit, but also with urine collection, resulted in increased urinary metanephrine concentrations, particularly in client-owned dogs compared to staff-owned dogs. In our study, a significant increase in pNMN, pMN and p3MT between T_1 and T_0 was found in healthy dogs. Although stress-related increases in plasma free metanephrines and 3MT were demonstrable, the results still fell within the RI, suggesting that environmental stress only marginally influences diagnostic testing for PCC.

Although a limitation of our study is that the number of dogs with NAI was small, dogs only were included if clinical signs were consistent with a diagnosis of PCC. This approach is in contrast to other studies, in which a variety of nonadrenal diseases were included.[13,14] Although any nonadrenal illness potentially can increase production of metanephrines, it is more relevant to specifically include patients with a clinical presentation similar to PCC, because doing so best reflects the situation in the clinical setting. One dog with NAI was treated with the calcium channel blocker amlodipine. In humans, amlodipine is associated with false-positive increases of plasma norepinephrine, but not pNMN and pMN.[36] In this particular dog, pMN was above the RI. Thus, it would be interesting to evaluate whether amlodipine affects plasma free metanephrines in dogs.

Glucocorticoids, produced locally by the adrenal cortex, are necessary for normal development and maintenance of the adrenal medulla. The expression of phenylethanolamine-N-methyltransferase, an enzyme responsible for converting norepinephrine to epinephrine, is dependent on glucocorticoids.[3,38] In humans, exogenous glucocorticoid administration may trigger pheochromocytoma crisis, a life-threatening condition with a massive release of catecholamines.[39] Because of this influence of glucocorticoids on catecholamine production and release in both normal chromaffin cells and PCC cells, dogs with HC were included in our study. Moreover, HC dogs were included because it is an important differential diagnosis for PCC because of the overlap in clinical signs, such as weakness, polyuria and polydipsia, tachypnea, and hypertension.[3,18] In our study, the 2 dogs with HC that had a pNMN concentration just above the RI were diagnosed with PDH and ADH. Because of the lack of adrenal histopathology in these 2 dogs, the presence of an additional PCC could not be ruled out. This lack of adrenal histopathology is the main limitation of our study. Three of 10 dogs diagnosed with ADH underwent adrenalectomy. Histopathology confirmed the presence of adrenocortical tumors in these dogs. In addition, histopathology of the adrenal glands was available in 4 of 10 dogs that had both pituitary and adrenal masses, confirming adrenocortical tumors. In the remaining 13 dogs, adrenal histopathology was not available. Although these dogs had history, physical examination, biochemical and hematological findings consistent with HC, and although there was no clinical suspicion of other adrenal pathology, definitive differentiation among a cortisol-secreting adrenocortical tumor, PCC, aldosteronoma, a nonfunctional adrenal mass or a metastastic mass was not possible. This consideration is especially important, because concurrent endocrine neoplasms, and specifically those affecting the pituitary, adrenomedullary or adrenocortical glands, are quite common in dogs.[40,41] It has been suggested that the increase in intra-adrenal cortisol, as seen in HC, may result in adrenal medullary hyperplasia and possibly neoplasia.[41]

In our study, the diagnosis of HC was based on lack of adequate cortisol suppression after an IV-LDDST or on increased UCCR. Although the reported specificity of the UCCR can be as low as 20% to 25%,[42,43] which warrants further testing and thus limits the use of

UCCR as a confirmatory test for HC in primary practice, several measures were taken in our study to eliminate factors that could decrease specificity and to ensure a definitive diagnosis of HC, such as: (1) the use of voided urine, collected at home minimally 2 days after a visit to a veterinary clinic; (2) all tested dogs were highly suspected to have HC, with compatible history, physical examination findings, laboratory abnormalities, and diagnostic imaging findings; (3) none of the dogs, in which UCCRs were used for the diagnosis of HC, had severe nonadrenal illness or concurrent disorders; and (4) an in-house radioimmunoassay, which uses anticortisol antibodies with little cross-reactivity to cortisol metabolites, was used for UCCR measurement, with an established decision threshold.

In conclusion, we report 95% RIs for plasma, urinary and salivary free metanephrines and 3MT. Using a cutoff based on the URLs showed that pNMN has high accuracy for the diagnosis of PCC, whereas pMN has moderate accuracy. The URL-based cutoffs for pNMN and pMN in our study are considerably lower than in previous studies, markedly increasing sensitivity while only minimally decreasing specificity for the diagnosis of PCC. The dog-specific RIs for urinary and salivary free metanephrines and 3MT, measured by LC-MS/MS, facilitate biochemical testing for PCC and further research in this area.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL USE DECLARATION

Authors declare no off-label use of antimicrobials.

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

Authors declare no IACUC or other approval was needed, as judged by the Utrecht University's DEC (Animal Ethics Committee). Signed informed consent was obtained from all owners prior to study participation.

HUMAN ETHICS APPROVAL DECLARATION

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

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SUPPORTING INFORMATION

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