

## Standard Article

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## Plasma N-Terminal Probrain Natriuretic Peptide, Vascular Endothelial Growth Factor, and Cardiac Troponin I as Novel Biomarkers of Hypertensive Disease and Target Organ Damage in Cats

E.S. Bijsmans, R.E. Jepson, C. Wheeler, H.M. Syme, and J. Elliott

**Background:** In the absence of ocular target organ damage (ocular-TOD), diagnosis of hypertension is challenging in cats. Biomarkers would provide additional support for the diagnosis of hypertension.

**Hypothesis:** Vascular endothelial growth factor (VEGF), N-terminal probrain natriuretic peptide (NT-proBNP), cardiac troponin I (cTnI), and urine protein-to-creatinine ratio (UPC) are predictors of systemic hypertension, will be increased in cats with hypertension with or without ocular-TOD, and will decrease with antihypertensive treatment.

**Methods:** Plasma VEGF, NT-proBNP, and cTnI concentrations and UPC were determined in healthy geriatric cats, normotensive cats with chronic kidney disease (CKD), hypertensive cats with evidence of hypertensive retinopathy (HT-ocular-TOD), and hypertensive cats without hypertensive ocular-TOD (HT-noTOD). Comparisons among groups were performed. Multivariable binary logistic regression models were built to identify independent biomarkers of hypertension and ocular-TOD. Receiver operator characteristic (ROC) curves were drawn to assess clinical use.

**Results:** Cats with HT-ocular-TOD had significantly higher VEGF than all other groups ( $P < .05$ ) and significantly higher NT-proBNP than healthy cats ( $P < .001$ ). Healthy cats had significantly lower cTnI than all other groups ( $P < .05$ ). No differences were found among groups for UPC ( $P = .08$ ). Cardiac troponin I and VEGF were independent predictors of hypertension ( $P < .05$ ), but none of the biomarkers were independent predictors of ocular-TOD. N-terminal probrain natriuretic peptide concentrations decreased with antihypertensive treatment ( $P < .001$ ). The ROC curves indicated that none of the biomarkers met the criteria to function as diagnostic tests for the diagnosis of hypertension or associated ocular-TOD.

**Conclusions and Clinical Significance:** Despite statistical significance and changes with ocular-TOD, antihypertensive treatment, or both, VEGF, NT-proBNP, and cTnI did not function as useful diagnostic tests for hypertension. Persistently increased systolic blood pressure (SBP) measurements in combination with funduscopy remains the preferred method for diagnosis of feline hypertension.

**Key words:** Chronic kidney disease; Hypertension.

White coat hypertension is reported to occur frequently in cats, with systolic blood pressure (SBP) increasing on average 22.3 mmHg during clinical examination, but with a highly unpredictable interindividual effect and SBP increasing by as much as 75 mmHg in some cats.<sup>1</sup> Without evidence of hypertensive retinopathy (ocular target organ damage [ocular-TOD]), it therefore can be challenging to diagnose hypertension, and clinicians must rely on documentation of consistently high SBP measurements in affected cats.<sup>2</sup> Hypertension has many immediate effects on the

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### Abbreviations:

CKD	chronic kidney disease
cTnI	cardiac troponin I
cTSH	canine thyroid-stimulating hormone
CV	coefficient of variation
GFR	glomerular filtration rate
HCM	hypertrophic cardiomyopathy
IRIS	International Renal Interest Society
NT-proBNP	N-terminal probrain natriuretic peptide
ROC	receiver operator characteristic
SBP	systolic blood pressure
TOD	target organ damage
UPC	urine protein-to-creatinine ratio
USG	urine specific gravity
VEGF	vascular endothelial growth factor

body and results in several physiologic responses in an attempt to decrease blood pressure. In hypertensive human patients, a number of these physiologic responses have been shown to involve substances that could function as biomarkers of hypertension, hypertensive TOD, efficacy of treatment, or some combination of these. The heart and blood vessels are considered target organs of hypertensive damage in both humans and cats.<sup>2</sup> Vascular endothelial growth factor (VEGF) secreted by endothelial cells and N-terminal pro-B-type natriuretic peptide (NT-proBNP) and cardiac troponin I (cTnI) secreted by cardiomyocytes are examples of biomarkers that have been investigated for use in

hypertensive human patients.<sup>3,4</sup> Urine protein-to-creatinine ratio (UPC) is a potential marker of TOD and renal injury.<sup>5,6</sup> Increased blood pressure leads to increased glomerular pressure and therefore leakage of protein into the urine. The UPC has been shown to be increased in hypertensive cats when compared to normotensive cats.<sup>7</sup>

Vascular endothelial growth factor is secreted in response to shear stress of endothelial cells<sup>8</sup> and mechanical stretch of vascular smooth muscle cells.<sup>9</sup> Plasma VEGF concentrations are increased in human patients with hypertension<sup>3,10</sup> and in patients with hypertensive retinopathy.<sup>11</sup> In addition, plasma VEGF decreases with antihypertensive treatment in humans.<sup>12</sup> Concentrations of NT-proBNP are increased in hypertensive human patients with peripheral arterial disease.<sup>4</sup> Human subjects with poor blood pressure control have higher plasma NT-proBNP concentrations, indicating its potential use as a marker for effectiveness of treatment.<sup>13</sup> Cats with hypertension and evidence of ocular-TOD have significantly higher NT-proBNP concentrations than do healthy cats and those with normotensive chronic kidney disease (CKD).<sup>14</sup> Hypertensive cats have been reported to have lower concentrations of NT-proBNP if hypertension is not accompanied by ocular-TOD, although these cats also had significantly lower blood pressure than did their counterparts with evidence of ocular changes.<sup>15</sup> Cardiac troponin I is produced only by cardiomyocytes and is a sensitive and specific marker of myocardial cell necrosis in humans<sup>16</sup> and dogs.<sup>17</sup> Cats with hypertrophic cardiomyopathy (HCM) have increased cTnI concentrations.<sup>18</sup> During hypoxic stress, cardiomyocytes release cTnI,<sup>16</sup> and it is possible that cTnI also is released when hypertension causes hypertrophy, stretch, strain, or damage. Human patients admitted to hospital for severe hypertension often have increased cTnI concentrations.<sup>19</sup>

The aims of our study were to assess UPC, VEGF, NT-proBNP, and cTnI as potential biomarkers of hypertension in cats with and without ocular-TOD, and to assess whether VEGF, NT-proBNP, and cTnI are informative in terms of assessment of blood pressure control.

## Materials and Methods

### *Clinics and Case Selection*

Cats diagnosed with hypertension at 2 first opinion practices in central London (People's Dispensary for Sick Animals in Bow and Beaumont Sainsbury Animal Hospital in Camden) between May 2007 and July 2015 were retrospectively identified. A standardized visit protocol was followed for each cat, which included full history and physical examination. A noninvasive Doppler technique<sup>a</sup> was used to measure SBP after a period of acclimatization. Average SBP was calculated from 5 consecutive readings after discarding the first reading. If average SBP was  $\geq 160$  mmHg, indirect fundoscopy was performed by the attending clinician with an independent light source and a lens, after applying 1 drop of tropicamide 1% to both eyes.

At the first visit to the clinic, informed owner consent was obtained to collect blood samples by jugular venipuncture and urine samples by cystocentesis. The standard clinic protocols,

information sheets, and consent forms for use of residual samples for research were approved by the Royal Veterinary College's Ethics and Welfare Committee. Blood samples were collected into lithium heparin tubes and EDTA tubes and held on ice (4°C) for a maximum of 6 hours before centrifugation<sup>b</sup> and separation. Plasma biochemistry was performed at an external laboratory.<sup>c</sup> Not all cats included in this study had serum total thyroxine (TT4) concentration measured, but cases were excluded if they had a TT4 > 40 nmol/L (reference range, 10–55 nmol/L), had clinical signs consistent with hyperthyroidism (eg, polyphagia, weight loss, palpable goiter, tachycardia), or were currently undergoing treatment for hyperthyroidism. Cats also were excluded if they had evidence of other concurrent disease (eg, palpation of an abdominal mass) and if they were on medications that were likely to alter blood pressure (eg, benazepril). Urine protein-to-creatinine ratios were determined at the same laboratory that was used for plasma biochemistry and TT4 measurements, and urine samples from cats with evidence of a urinary tract infection were excluded from analysis.

Four groups were included: cats diagnosed with azotemic CKD (CKD), healthy geriatric cats (Healthy), hypertensive cats with evidence of ocular hypertensive TOD (HT-ocular-TOD), and hypertensive cats without evidence of hypertensive retinopathy (HT-noTOD). The hypertensive visit for the HT-ocular-TOD and HT-noTOD groups and 1 visit for the CKD and healthy groups were used for the cross-sectional analyses. Azotemic CKD was diagnosed based on a plasma creatinine concentration  $\geq 2.0$  mg/dL either on 2 consecutive visits or on a single occasion if in conjunction with urine specific gravity (USG) <1.035. Healthy cats had unremarkable history and physical examination, SBP <160 mmHg, and plasma biochemistry lacked abnormalities deemed to be clinically relevant by the attending clinician. Cats that had urine samples available and USG <1.035 were only included as healthy if they had follow-up visits at which the cat was assessed as healthy by the attending clinician and no azotemia developed within 6 months. All cats included in the HT-ocular-TOD group had SBP  $\geq 160$  mmHg and evidence of hypertensive retinopathy or choroidopathy on indirect ophthalmologic examination, whereas all cats included in the HT-noTOD group were diagnosed based on a SBP  $\geq 170$  mmHg on 2 consecutive visits, but did not have evidence of ocular hypertensive TOD. A small number of the cats had undergone echocardiographic assessment, but to prevent bias in the selection, the authors were blinded to the results of the cardiac evaluation.

Only hypertensive cats were included in the longitudinal study. All hypertensive cats were treated with amlodipine besylate.<sup>d</sup> A blood sample was taken at the time of diagnosis of hypertension (included in the cross-sectional study), at the first visit antihypertensive control was achieved (defined as SBP <160 mmHg and receiving 0.625–1.25 mg amlodipine/cat/day) and approximately 4 months later (approximately 5 months after diagnosis of hypertension). All cats must have had residual stored heparinized plasma and EDTA plasma samples available for all visits included in both the cross-sectional and longitudinal studies to be eligible for inclusion.

### *Measurement of UPC, VEGF, NT-proBNP, and cTnI*

The VEGF assay validated in this study was a commercially available canine VEGF ELISA.<sup>e</sup> Precision and reproducibility of the assay for heparinized feline plasma samples were determined by calculating intra- and interassay coefficients of variation (CV). Dilutional parallelism was assessed, as was the effect of 4 freeze-thaw cycles.

Plasma EDTA samples previously stored at  $-80^{\circ}\text{C}$  were used for batch analysis of NT-proBNP and cTnI at a commercial

laboratory.<sup>c</sup> Batch analysis of UPC on urine samples previously stored at  $-80^{\circ}\text{C}$  was performed at the same laboratory. The UPC was only determined for samples included in the cross-sectional study, and not post-treatment with amlodipine in the hypertensive groups.

### Statistical Analyses

Statistical analyses were performed by 2 different statistical software packages.<sup>f</sup> Results are presented as median [25th, 75th percentiles], and statistical significance was determined as  $P < .05$ . Variables were assessed for normality by visual inspection of histograms. The number of cats that was needed for the cross-sectional study was estimated by a parametric power calculation on unpublished VEGF data on healthy cats and hypertensive cats without ocular-TOD, and estimated data for cats with ocular-TOD, with power set at 80% and significance at 5%. This power calculation was repeated after all measurements were performed, and an actual mean concentration was known for ocular-TOD cats. The parametric power calculation was adjusted for the fact that the data were skewed by multiplying it by 1.15 (15% additional cats).

Cross-sectional comparisons among HT-ocular-TOD, HT-noTOD, CKD, and healthy cats were made using Kruskal–Wallis tests with posthoc comparisons by Dunn’s test or a 1-way ANOVA with posthoc Tukey’s test. The proportion of cats with concurrent CKD was compared between HT-ocular-TOD and HT-noTOD using Fisher’s exact test. Comparisons among related visits within the hypertensive groups were made using the non-parametric Wilcoxon signed-rank test and Friedman test with posthoc comparisons using the Bonferroni method.

To identify predictors of hypertension, HT-ocular-TOD and HT-noTOD were grouped as hypertensive, and the healthy cats and cats with CKD functioned as the control group. To identify predictors of ocular-TOD, the HT-noTOD cats functioned as the control group and were compared to the cats with HT-ocular-TOD. Plasma VEGF, NT-proBNP, and cTnI concentrations and UPC all were included as explanatory variables in univariable binary logistic regression models with hypertension or ocular-TOD as dependent variable. All variables were log-transformed to meet normality criteria. Variables significant at the 20% level were included in a multivariable binary logistic regression to identify the biomarkers that were independent predictors of hypertension and ocular-TOD.

Receiver operating characteristic curves (ROC) were used to determine whether VEGF, NT-proBNP, cTnI, and UPC could be used to predict hypertension and ocular-TOD in a clinical setting. Sensitivity and specificity were used to describe potential cutoffs for the variables that had an area under the curve (AUC) significantly  $>0.5$ . The combined use of VEGF, NT-proBNP, and cTnI to diagnose hypertension or ocular-TOD was explored. The cutoff value for each variable was determined based on the greatest combined sensitivity and specificity. Sensitivity and specificity were determined using each possible combination of the significant values to diagnose hypertension and ocular-TOD.

## Results

### Validation of the Canine VEGF ELISA for Feline Plasma

The intra-assay CVs for samples measuring 99.5, 100.9, and 101.5 pg/mL were 9.9, 5.0, and 4.5%, respectively. The interassay CVs for samples measuring 63.2, 100.3, and 109.5 pg/mL were 13.9, 7.2 and 4.3%, respectively. Dilutional parallelism was demonstrated:

94.9 ( $\pm 13.6$ ) % recovery. Measurement after 4 freeze-thaw cycles gave a recovery of 119.4 ( $\pm 4.3$ ) % ( $n = 3$ ).

### Subjects

The power calculation indicated 25 cats were needed per group. A total of 25 hypertensive cats with ocular-TOD, 24 hypertensive cats without ocular-TOD, 25 healthy cats, and 25 CKD cats were included (Table 1). As expected, both hypertensive groups had significantly higher SBP than did the healthy and CKD cats. The healthy cats were significantly younger and had lower plasma urea and creatinine concentrations, and higher USG than all other groups. Three healthy cats had USG  $<1.035$  at the included visit. All 3 had follow-up visits at which they remained nonazotemic and were deemed healthy by the attending veterinarian. Two of these cats had urine samples available at follow-up visits, and both had concentrated urine at those visits. Twenty-nine cats had NT-proBNP  $>270$  pmol/L, which makes cardiac disease more likely ([http://www.idexx.co.uk/pdf/en\\_gb/smallanimal/reference-laboratories/clinical-guidance-cardiac-disease.pdf](http://www.idexx.co.uk/pdf/en_gb/smallanimal/reference-laboratories/clinical-guidance-cardiac-disease.pdf), downloaded on 21/09/2015). Twelve cats with NT-proBNP  $>270$  pmol/L had an echocardiographic result available, of which 4 were classified as normal (1 in the HT-ocular-TOD group; 3 in the CKD group), 5 had evidence of severe hypertrophy (3 in the HT-noTOD group, all were already on amlodipine treatment at the time of echocardiography; 2 had CKD), 1 had pseudohypertrophy likely because of dehydration (HT-ocular-TOD group, echocardiography was performed on the day hypertension was diagnosed), 1 had possible left ventricular hypertrophy (CKD group), and 1 cat had dynamic left ventricular outflow tract obstruction (HT-noTOD group, echocardiography performed on the day hypertension was diagnosed). The majority of cats ( $n = 88$ ) did not have an echocardiographic assessment available.

### Vascular Endothelial Growth Factor, NT-proBNP, cTnI, and UPC as Predictors of Hypertension and Ocular-TOD

**Univariable Analyses.** Comparison among groups determined that HT-ocular-TOD had significantly higher plasma VEGF concentrations than did HT-noTOD, CKD, and healthy cats ( $P < .05$ ; Fig 1). The power calculation with the actual values indicated that 35 cats were needed per group to show a potential difference between HT-ocular-TOD and HT-noTOD. A sample size of 58 per group would be needed to detect a decrease of 20% in response to treatment when grouping HT-ocular-TOD and HT-noTOD, assuming a standard deviation (SD) of 46 on the change in VEGF with treatment (80% power with significance set at 5%). Univariable binary logistic regression indicated that log-transformed plasma VEGF concentration was a positive predictor of hypertension ( $P < .05$ ; Table 2). The ROC curve indicated that plasma VEGF could be used to predict hypertension (AUC, 0.64;  $P = .016$ )

**Table 1.** Clinicopathologic variables for healthy, CKD, HT-ocular-TOD, and HT-noTOD cats.

	Healthy (n = 25)	CKD (n = 25)	HT-noTOD (n = 24)	HT-Ocular-TOD (n = 25)
VEGF (pg/mL)	49.4 [39.0, 94.4] <sup>a</sup>	70.4 [51.2, 78.5] <sup>a</sup>	53.3 [39.9, 106.6] <sup>a</sup>	105.6 [77.3, 142.5] <sup>b</sup>
NT-proBNP (pmol/L)	67.0 [28.5, 118.0] <sup>a</sup>	126.0 [75.0, 290.5] <sup>ab</sup>	120.0 [75.0, 289.0] <sup>ab</sup>	236.0 [114.5, 519.0] <sup>b</sup>
cTnI (ng/mL)	0.02 [0.01, 0.03] <sup>a</sup>	0.04 [0.02, 0.05] <sup>b</sup>	0.04 [0.02, 0.06] <sup>b</sup>	0.07 [0.05, 0.14] <sup>b</sup>
SBP (mmHg)	131.2 [113.0, 139.4] <sup>a</sup>	136.0 [133.2, 141.4] <sup>a</sup>	179.4 [175.3, 188.2] <sup>b</sup>	184.8 [168.4, 203.6] <sup>b</sup>
UPC	0.18 [0.13, 0.22]	0.24 [0.15, 0.58]	0.25 [0.20, 0.50]	0.17 [0.14, 0.30]
Heart rate (bpm)	180 [165, 199]	192 [180, 210]	180.0 [180.0, 204.0]	182 [164, 205]
Age (years)	12.1 [11.1, 13.2] <sup>a</sup>	15.3 [12.4, 16.7] <sup>ab</sup>	15.3 [12.8, 17.0] <sup>b</sup>	15.8 [13.5, 18.0] <sup>b</sup>
Weight (kg)	4.5 [3.8, 5.4] <sup>a</sup>	3.8 [3.3, 4.6] <sup>bc</sup>	4.6 [3.5, 5.7] <sup>ab</sup>	3.6 [3.0, 4.4] <sup>c</sup>
PCV (%)	36 [34, 40]	35 [29, 39]	37 [34, 40]	35 [30, 39]
Albumin (g/dL)	3.2 [3.0, 3.3]	3.1 [3.0, 3.2]	3.3 [3.1, 3.4]	3.1 [2.9, 3.3]
Creatinine (mg/dL)	1.4 [1.3, 1.6] <sup>a</sup>	2.4 [2.1, 2.9] <sup>b</sup>	2.1 [1.7, 2.9] <sup>b</sup>	2.3 [1.6, 2.8] <sup>b</sup>
Urea (mmol/L)	9.6 [8.1, 11.4] <sup>a</sup>	15.0 [12.5, 19.2] <sup>b</sup>	15.8 [11.8, 21.6] <sup>b</sup>	16.2 [11.4, 22.3] <sup>b</sup>
Phosphate (mg/dL)	3.56 [3.25, 4.15]	3.62 [3.28, 4.30]	4.09 [3.34, 4.74]	3.84 [3.34, 4.92]
Total calcium (mg/dL)	9.8 [9.5, 10.2]	10.4 [9.8, 11.1]	10.0 [9.8, 10.4]	10.0 [9.5, 10.6]
Sodium (mEq/L)	153.7 [152.5, 154.7]	155.8 [153.5, 157.6]	154.1 [151.3, 156.5]	154.2 [152.3, 156.7]
Potassium (mEq/L)	4.1 [3.7, 4.3]	4.1 [3.8, 4.3]	4.1 [3.7, 4.5]	4.0 [3.7, 4.3]
Chloride (mEq/L)	119.6 [118.2, 122.5] <sup>a</sup>	119.7 [118.1, 120.9] <sup>ab</sup>	117.5 [115.3, 119.7] <sup>b</sup>	118.8 [116.3, 120.3] <sup>ab</sup>
Cholesterol (mmol/L)	181.5 [142.9, 212.4]	223.9 [181.5, 278.0]	202.3 [177.6, 239.4]	200.8 [179.5, 219.3]
USG	1.047 [1.036, 1.057] <sup>a</sup>	1.018 [1.017, 1.024] <sup>b</sup>	1.018 [1.015, 1.028] <sup>b</sup>	1.019 [1.016, 1.025] <sup>b</sup>

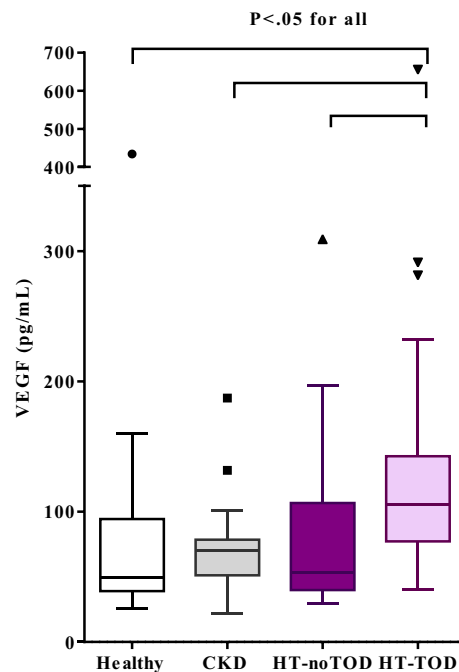
CKD, chronic kidney disease; HT-ocular-TOD, hypertensive cats with evidence of ocular target organ damage; HT-noTOD, hypertensive cats without evidence of ocular target organ damage; VEGF, vascular endothelial growth factor; NT-proBNP, N-terminal probrain natriuretic peptide; cTnI, cardiac troponin I; UPC, urine protein-to-creatinine ratio; SBP, systolic blood pressure; PCV, packed cell volume; USG, urine specific gravity.

Comparisons between groups were performed using a Kruskal–Wallis with posthoc Dunn's test or 1-Way ANOVA with posthoc Tukey's test. Medians bearing the same superscript letter are not significantly different from one another. All values are presented as median [25th, 75th percentiles].

with an optimal value of >76.1 pg/mL (specificity, 72%; sensitivity, 59%). Concentrations of VEGF were found to be a positive and significant predictor of ocular-TOD in the univariable analysis ( $P = .01$ ; Table 3). The cutoff to diagnose ocular-TOD was defined at >71.6 pg/mL (AUC, 0.73; sensitivity, 80%; specificity, 62.5%;  $P < .01$ ).

Plasma NT-proBNP concentration was found to be significantly higher in HT-ocular-TOD when compared with healthy cats ( $P < .001$ ; Fig 2). Log-transformed plasma NT-proBNP concentration was a positive predictor of hypertension in the univariable analysis ( $P < .01$ ; Table 2). The optimum cut-point for NT-proBNP to predict hypertension was >134.5 pmol/L (AUC, 0.68; sensitivity, 59%; specificity, 72%;  $P = .002$ ). Log-transformed NT-proBNP was not a significant predictor of ocular-TOD in the univariable analysis ( $P = .167$ ; Table 3). The ROC had an AUC that was not significantly different from the line of zero discrimination (AUC = 0.64,  $P = .08$ ). A cutoff point for using NT-proBNP to predict ocular-TOD therefore was not determined.

Plasma cTnI concentration was significantly lower in healthy cats when compared with HT-ocular-TOD, HT-noTOD, and CKD (Fig 3). Log-transformed cTnI was a positive significant predictor of hypertension in the univariable analysis ( $P < .001$ ; Table 2). The ROC curve indicated that the cutoff value for cTnI to determine hypertension to be >0.045 ng/mL (AUC, 0.73; sensitivity, 63%; specificity, 78%;  $P < .0001$ ). Plasma cTnI concentration was significantly associated with



**Fig 1.** Plasma vascular endothelial growth factor (VEGF) concentration in healthy cats (n = 25), cats with chronic kidney disease (n = 25), hypertensive cats without evidence of hypertensive ocular target organ damage (HT-noTOD) (n = 24), and hypertensive cats with evidence of hypertensive retinopathy (HT-ocular-TOD) (n = 25). HT-ocular-TOD had significantly higher plasma VEGF concentration than all other groups ( $P < .05$  for all comparisons).

**Table 2.** Univariable analysis of VEGF, NT-proBNP, and cTnI as predictors of hypertension in cats.

Variable	n	$\beta$	SE	P-Value	95% CI for $\beta$
log(cTnI)	99	0.814	0.237	.0006	0.375 to 1.311
log(NT-proBNP)	99	0.565	0.200	.0046	0.190 to 0.978
log(VEGF)	99	0.843	0.351	.0164	0.185 to 1.574
log(UPC)	50	0.310	0.429	.4700	-0.519 to 1.199

VEGF, vascular endothelial growth factor; NT-proBNP, N-terminal probrain natriuretic peptide; cTnI, cardiac troponin I; UPC, urine protein-to-creatinine ratio; n, number of cats; 95% CI, 95% confidence interval.

Log(cTnI), log(NT-proBNP), and log(VEGF) were significant at the 20% level and included in the multivariable analysis.

**Table 3.** Univariable analysis of VEGF, NT-proBNP, and cTnI as predictors of TOD in cats.

Variable	n	$\beta$	SE	P-Value	95% CI for $\beta$
log(VEGF)	49	1.405	0.546	.01	0.426 to 2.594
log(cTnI)	49	0.770	0.350	.028	0.136 to 1.528
log(UPC)	27	1.034	0.660	.118	-0.153 to 2.518
log(NT-proBNP)	49	0.3853	0.279	.167	-0.145 to 0.966

VEGF, vascular endothelial growth factor; NT-proBNP, N-terminal probrain natriuretic peptide; cTnI, cardiac troponin I; UPC, urine protein-to-creatinine ratio; n, number of cats; 95% CI, 95% confidence interval.

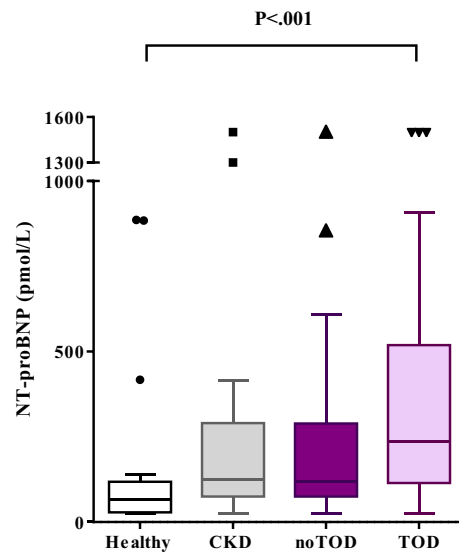
All 4 variables were significant at the 20% level and included in the multivariable analysis.

ocular-TOD in the univariable analysis ( $P = .028$ ; Table 3). The drawn ROC curve indicated that cTnI could be used to predict ocular-TOD, with a cutoff value of  $>0.045$  pg/mL (AUC = 0.71; sensitivity, 84%; specificity, 58%;  $P = .01$ ).

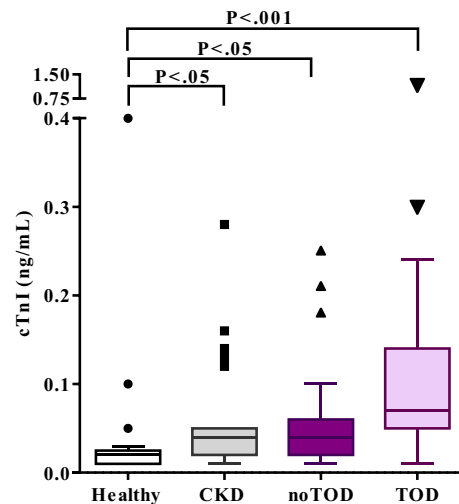
The UPC was not found to be different among groups (Fig 4). Log-transformed UPC was not a significant predictor of hypertension in the univariable analysis ( $P = .47$ ; Table 2). The ROC for determination of hypertension therefore was not drawn. The UPC was associated with ocular-TOD at the 20% level in the univariable analysis ( $P = .118$ ; Table 3). However, the ROC curve indicated that UPC could not be used to predict ocular-TOD (AUC = 0.67,  $P = .14$ ).

**Multivariable Analyses.** Plasma VEGF, NT-proBNP, and cTnI concentrations all were predictive of hypertension at the 20% level in the univariable logistic regression and were included in the multivariable model. This indicated that log-transformed plasma VEGF and cTnI concentrations remained significant and positive predictors of hypertension (Table 4). A 10-fold increase in plasma VEGF concentration was associated with an odds ratio (OR) of 2.16 (95% confidence interval [CI], 1.06–4.65;  $P < .05$ ), and a 10-fold increase in plasma cTnI concentration had an OR of 2.38 (95% CI, 1.17–5.28;  $P < .05$ ).

All 4 variables were significant at the 20% level to predict ocular-TOD and therefore were included in the



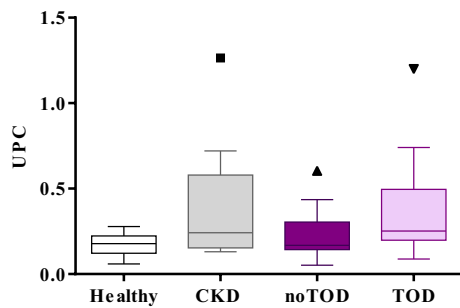
**Fig 2.** Plasma N-terminal probrain natriuretic peptide (NT-proBNP) concentrations in healthy cats, ( $n = 25$ ), cats with chronic kidney disease ( $n = 25$ ), hypertensive cats without evidence of hypertensive ocular target organ damage (HT-noTOD) ( $n = 24$ ), and hypertensive cats with evidence of hypertensive retinopathy (HT-ocular-TOD) ( $n = 25$ ). HT-TOD had significantly higher plasma NT-proBNP concentration than healthy cats ( $P < .001$ ).



**Fig 3.** Plasma cardiac troponin I (cTnI) concentrations in healthy cats, ( $n = 25$ ), cats with chronic kidney disease ( $n = 25$ ), hypertensive cats without evidence of hypertensive ocular target organ damage (HT-noTOD) ( $n = 24$ ), and hypertensive cats with evidence of hypertensive retinopathy (HT-ocular-TOD) ( $n = 25$ ). Healthy cats had significantly lower plasma cTnI concentrations than all other groups ( $P < .05$ ).

multivariable analysis. None of the variables remained significantly associated with ocular-TOD (Table 5).

The ROC curves indicated that cTnI had the highest specificity for diagnosing hypertension (78%). The UPC could not reliably diagnose hypertension, and only combinations of cTnI, VEGF, and NT-proBNP were



**Fig 4.** Urine protein-to-creatinine ratio (UPC) in healthy cats, (n = 13), cats with chronic kidney disease (n = 10), hypertensive cats without evidence of hypertensive ocular target organ damage (HT-noTOD) (n = 14), and hypertensive cats with evidence of hypertensive retinopathy (HT-ocular-TOD) (n = 13). No significant differences were found between groups.

**Table 4.** Multivariable analysis including VEGF, NT-proBNP, and cTnI to identify independent predictors of hypertension in cats.

Variable	$\beta$	SE	P-Value	95% CI for $\beta$
log(cTnI)	0.866	0.380	.023	0.157 to 1.663
log(VEGF)	0.768	0.373	.04	0.059 to 1.536
log(NT-proBNP)	-0.102	0.334	.761	-0.777 to 0.546
(Intercept)	0.006	2.970	.998	-5.815 to 5.958

cTnI, cardiac troponin I; NT-proBNP, N-terminal probrain natriuretic peptide; VEGF, vascular endothelial growth factor; n, number of cats; 95% CI, 95% confidence interval.

Both cTnI and VEGF remained independent positive predictors of hypertension in the multivariable analysis.

explored. Using a combination of VEGF and either NT-proBNP or cTnI, specificity of 94% could be achieved, with sensitivity of 37% and 45%, respectively. The specificity could be increased to 96% by incorporating all 3 variables, which was associated with a decrease in sensitivity to 31%.

Assessment of the ROC curves indicated that NT-proBNP concentration and UPC could not reliably be used to diagnose ocular-TOD, and therefore, only the combination of VEGF and cTnI was explored for this purpose. Plasma cTnI concentration  $>0.045$  pg/mL in combination with plasma VEGF concentration  $>71.6$  pg/mL had a specificity of 88% to diagnose ocular-TOD; sensitivity was 76%.

**Changes in VEGF, NT-proBNP, and cTnI With Treatment of Hypertension.** Cats with HT-ocular-TOD had received amlodipine for  $20 \pm 8$  days, and HT-noTOD for  $25 \pm 15$  days at the first controlled visit. The third visit was  $141 \pm 43$  days after starting treatment for the HT-ocular-TOD group and  $157 \pm 42$  days for the HT-noTOD group. At the diagnosis of hypertension, 20 cats in the HT-ocular-TOD group and 16 cats in the HT-noTOD group had concurrent CKD ( $P = .35$ ). Plasma creatinine concentration did not significantly change over the course of 5 months ( $P = .58$ ), and no cats showed evidence of CKD progression (defined as a  $>25\%$  increase in plasma creatinine concentration).

**Table 5.** Multivariable analysis including VEGF, NT-proBNP, and cTnI to identify independent predictors of TOD in cats.

Variable	$\beta$	SE	P-Value	95% CI for $\beta$
log(VEGF)	1.520	0.794	.056	0.155 to 3.389
log(cTnI)	1.526	0.970	.115	-0.150 to 3.768
log(NT-proBNP)	-0.424	0.736	.564	-1.956 to 1.038
log(UPC)	0.428	0.802	.594	-1.076 to 2.224
(Intercept)	0.113	6.688	.987	-13.96 to 13.25

VEGF, vascular endothelial growth factor; cTnI, cardiac troponin I; NT-proBNP, N-terminal probrain natriuretic peptide, UPC, urine protein-to-creatinine ratio; 95% CI, 95% confidence interval; TOD, target organ damage.

No independent positive or negative predictors of ocular-TOD were found.

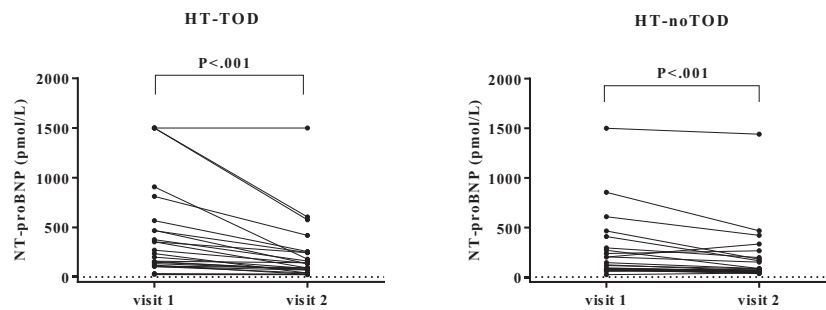
At the hypertensive visit, median plasma VEGF concentration in the HT-ocular-TOD group was 105.6 [77.30, 142.5] pg/mL. At the first controlled visit, the median concentration was 91.0 [64.2, 162.7] pg/mL, and at visit 3, the concentration was 86.2 [64.0, 124.7] pg/mL. No significant change over time was found ( $P = .07$ ). The VEGF concentration for the HT-noTOD group was 53.3 [39.9, 106.6] pg/mL at baseline, and 62.4 [45.8, 105.3] pg/mL at the first controlled visit, followed by a concentration of 54.3 [38.6, 105.3] at visit 3 (not significant,  $P = .19$ ).

Because of financial considerations, NT-proBNP concentrations from both the hypertensive visit and the first controlled visit were determined initially, before making a decision with regard to measuring the sample concentration of a visit after approximately 5 months of amlodipine treatment. In HT-ocular-TOD, NT-proBNP concentration decreased significantly with approximately 2 weeks of amlodipine treatment, from 236.0 [114.5, 519.0] pmol/L to 94.0 [56.0, 245.5] pmol/L ( $P < .001$ ). The NT-proBNP concentration also significantly decreased in the HT-noTOD-group, from 120.0 [75.0, 289.0] pmol/L to 78.0 [55.0, 192.8] pmol/L ( $P < .001$ ; Fig 5). Because NT-proBNP showed a significant decrease at the first controlled visit, this marker was not measured after 5 months of antihypertensive treatment.

Plasma cTnI concentration did not significantly change with antihypertensive treatment in either group (HT-ocular-TOD: hypertensive visit, 0.07 [0.05, 0.14] ng/mL; first controlled visit, 0.06 [0.04, 0.12] ng/mL; after approximately 5 months of treatment, 0.07 [0.05, 0.13] ng/mL;  $P = .25$ ); HT-noTOD: hypertensive visit, 0.04 [0.02, 0.06] ng/mL; first controlled visit, 0.04 [0.03, 0.11] ng/mL; after approximately 5 months of treatment, 0.05 [0.03, 0.08] ng/mL;  $P = .48$ ).

## Discussion

Plasma VEGF and cTnI concentrations were found to be independent predictors of hypertension. Plasma NT-proBNP decreased significantly with antihypertensive treatment. These results seem promising for the use



**Fig 5.** Plasma N-terminal probrain natriuretic peptide (NT-proBNP) in hypertensive cats at visit hypertension was diagnosed (visit 1) and first controlled visit on amlodipine treatment (defined as systolic blood pressure below 160 mmHg) (visit 2; for HT-ocular-TOD after  $20 \pm 8$  days and HT-noTOD  $25 \pm 15$  days). The median NT-proBNP concentration decreased by 60% in cats with evidence of hypertensive ocular target organ damage (HT-ocular-TOD) and by 35% in cats without evidence of hypertensive retinopathy (HT-noTOD).

of these markers for the diagnosis and management of hypertension, but the ROC curves indicated that all markers evaluated had fairly low sensitivity. Additionally, all groups had substantial overlap in VEGF, NT-proBNP, and cTnI concentrations, making the markers less able to differentiate among groups. The power calculation that was performed using the VEGF concentrations indicated that a larger sample size could have led to significant results. The cats included in the current study were diagnosed with hypertension based on either high SBP with evidence of ocular-TOD or had persistently high SBPs  $>170$  mmHg. The groups were chosen in such a way to be able to identify markers of definitive hypertension and ocular-TOD.<sup>2</sup> The repeatedly high SBP in some cats without ocular-TOD may or may not have been because of white coat hypertension, which is a limitation that is challenging to manage. Use of biomarkers would therefore be an attractive alternative to the current way of diagnosing hypertension by repeated blood pressure measurements.

The UPC previously has been shown to be increased in hypertensive cats.<sup>7</sup> Additionally, UPC can be increased in CKD and is a predictor of CKD progression.<sup>6</sup> The fact that most of the hypertensive cats included in the current study had concurrent CKD therefore may be an explanation for why this marker was not found to be a significant predictor of hypertension or ocular-TOD. Only half of the included cats had a palpable bladder and therefore a urine sample available for UPC measurement. The smaller sample size therefore also could explain the lack of significance. However, the UPC distribution was fairly atypical, with 25% of normotensive CKD cases having UPC of 0.58, and 25% of healthy cats having UPC  $\geq 0.22$ . Why this distribution occurred in the current group is unclear, but possibly because of the low number of cats. None of the included cases had urinary tract infection.

One consideration is the speed with which the concentrations of these markers change in response to increases in blood pressure in cats, which currently is unknown.<sup>20</sup> The rate of elimination of the biomarkers also must be considered, especially because many cats included in this study had concurrent CKD, which may decrease the clearance of VEGF,<sup>21</sup> NT-proBNP,<sup>14,22,23</sup>

and cTnI.<sup>24,25</sup> For this reason, both healthy and CKD cats were included as control groups. Increased plasma VEGF concentrations have been reported in human patients with CKD when compared to healthy controls, and concentrations were higher with more advanced CKD.<sup>26,27</sup> In our study, large overlap was found among plasma VEGF concentrations of hypertensive cats, healthy cats, and cats with CKD, but cats with CKD did not have significantly higher plasma VEGF concentrations compared to healthy cats. The concentration of NT-proBNP also has been reported to increase with decreasing glomerular filtration rate (GFR) in some species.<sup>28</sup> Cats with CKD included in our study did not have significantly higher NT-proBNP concentrations than did healthy cats, which conflicts with previously published results.<sup>14</sup> However, in the previous study, the cats with CKD were in more advanced International Renal Interest Society (IRIS) stages. The majority of cats in our study were diagnosed in IRIS stage 2, which could explain the absence of significant differences among the groups. The cTnI concentrations were higher in CKD, HT-ocular-TOD, and HT-noTOD cats when compared to the healthy group. This difference is likely to be because of underlying CKD in the hypertensive cats. Previous studies that evaluated cTnI in cats with CKD have reported concentrations above the reference interval.<sup>29</sup> Human patients with renal failure often have increased cTnI concentrations.<sup>30</sup> However, it is possible that cats with CKD, like human patients, develop concurrent heart disease or suffer from uremic damage to the heart, and whether the increase found in our study is caused by increased production or decreased excretion warrants further research.

It seems unlikely that any of the biomarkers studied would change measurably because of the transient increases in blood pressure that occur during a visit to the clinic. Increases in VEGF occur rapidly in response to stretch, deformation, and shear stress in cell culture,<sup>31</sup> but take up to 3 days to increase in animals with hypertension.<sup>32,33</sup> Brain natriuretic peptide (BNP) gene expression and mRNA levels increase rapidly (within 2 hours) with stretch and pressure overload, and BNP gene expression increases after 2 hours of stretch in mouse cardiac tissue specimens.<sup>34</sup> The N-terminal

fragment of BNP (NT-proBNP) is spliced off after BNP secretion, and, because elimination of NT-proBNP is much slower, it is considered a more suitable assessment method for change in BNP in clinical patients. Because of the slow elimination, the concentration in the blood will reflect prevailing concentrations over a much longer period than the active hormone.<sup>35</sup> When inducing necrosis in cell culture studies, cTnI release does not occur until the cardiomyocytes are irreversibly damaged (after >18 hours), whereas in the reversible phase, the concentrations do not increase.<sup>36</sup> A few studies have described cTnI release from viable cardiomyocytes in response to stretch, indicating that myocardial strain rather than cell injury could lead to cTnI increases.<sup>37</sup> Overall, however, the evidence suggests that increases in cTnI do not occur rapidly with transient increases in SBP.

In our study, VEGF appeared to hold the greatest promise as a marker of ocular-TOD caused by hypertension, but the marker did not prove to be significant in the multivariable analysis. Plasma VEGF concentration was not increased in cats that were diagnosed with hypertension in the absence of ocular-TOD. Vascular endothelial growth factor has been proposed as a marker for hypertension in humans, but it is not yet clear whether it is a marker of, or a player in, the development of hypertension. Hypertensive human patients have increased plasma VEGF concentrations compared to normotensive controls.<sup>10,12,38,39</sup> Plasma VEGF concentrations may increase with increasing pressure, and the development of ocular-TOD could simply be caused by higher pressure in the arteries and arterioles of target organs. However, the SBP of cats without ocular-TOD was not significantly lower than the SBP of cats with ocular-TOD. It therefore seems more likely that those cats with ocular-TOD have a different pathophysiologic response to increased pressure than cats without ocular-TOD or that the underlying pathophysiology for the development of hypertension is different. Considering the edematous ocular lesions often seen in hypertensive cats,<sup>2</sup> a permeability factor like VEGF may play a role in the development of retinopathy and choroidopathy. However, if a systemic response was damaging to the retina, the damage is likely also to occur in other target organs, such as the kidney, and cats with hypertensive ocular-TOD did not have significantly higher plasma creatinine concentrations or UPC than those without. It therefore also is possible that certain cats in the HT-noTOD group were incorrectly classified and that both groups suffered similar hypertensive damage to their kidneys.

If deranged angiogenesis underlies the hypertension observed in cats with ocular-TOD, it could be accompanied by increased plasma VEGF concentrations.<sup>40</sup> In human patients, VEGF has been reported to decrease with a comparable duration of antihypertensive treatment as that studied here.<sup>38</sup> Plasma VEGF concentration did not decrease with effective antihypertensive treatment in the cats in our study, something that would have been expected to happen if VEGF was increased as a physiologic response. This observation

could support a role for VEGF in the causation of hypertensive ocular-TOD in cats. Additional studies including a larger number of cats, both before and after they develop hypertension with and without ocular-TOD, are required to investigate this hypothesis.

Our study is the second to assess NT-proBNP concentrations in hypertensive cats.<sup>14</sup> Other studies published to date have evaluated NT-proBNP as a biomarker of myocardial disease,<sup>41</sup> and for differentiation of cardiac and noncardiac causes of respiratory distress.<sup>41</sup> The concentration of NT-proBNP was not found to be an independent predictor of hypertension or ocular-TOD in the cats in our study, although many cats had increased concentrations, including in the control groups. This finding could be a result of subclinical heart disease, which is very prevalent in the feline population.<sup>42,43</sup> Up to 16% of cats have subclinical cardiomyopathy,<sup>44</sup> and therefore, exclusion of cats with evidence of heart disease may have introduced bias.<sup>45</sup> Furthermore, echocardiography cannot differentiate between primary heart disease and secondary hypertrophy because of hypertension. Additionally, not having the opportunity to perform echocardiography on all cats is representative of general practice. It was therefore decided to keep all cats, including those with evidence of severe hypertrophy on echocardiographic assessment, with or without an increased NT-proBNP concentration, in the study. Additionally, thyroid status was assessed in all cats based on history and clinical findings, but was not always based on TT4 measurement. An important consideration in clinical practice is that NT-proBNP concentrations are significantly increased in hyperthyroidism.<sup>46,47</sup>

Limitations of our study that must be recognized are that cats with primary cardiac disease with or without occult hyperthyroidism could have been included. Target organ damage was determined by fundic examination. However, the eye is not the only organ susceptible to hypertensive damage. The kidneys, brain, and heart also are possible targets, most cats included in the HT-noTOD group had CKD, and 3 cats had evidence of severe hypertrophy on echocardiography. It is not possible to differentiate hypertrophy as a consequence of primary cardiac disease versus that secondary to the effects of systemic hypertension, and therefore, the cardiac changes identified on echocardiography may have reflected TOD. For this reason, 2 separate analyses were performed—1 with the hypertensive cats grouped together and 1 with the ocular-TOD cats separately.

A few studies describe NT-proBNP as a marker of TOD in human medicine.<sup>4,48</sup> Hypertensive cats with ocular-TOD had significantly higher plasma concentrations NT-proBNP than did healthy cats, but HT-ocular-TOD cats did not have significantly different NT-proBNP from HT-noTOD. Studies of humans have reported similar results when comparing BNP and NT-proBNP in patients with hypertension and TOD to controls.<sup>49</sup> The finding that HT-noTOD and HT-ocular-TOD did not differ significantly is in contrast with results previously presented,<sup>15</sup> and might be explained by the absence of a difference in SBP between



the 2 groups included in our study. The concentration of NT-proBNP significantly decreased in both hypertensive groups with antihypertensive treatment, suggesting that it may be useful to assess the efficacy of treatment. Studies following cats over a longer period of time and comparing animals in different SBP categories over time would be a useful next step.

Cardiac troponins (cTns) historically have been accepted as markers for cardiac injury and myocardial cell necrosis.<sup>36,50</sup> In cats included in our study, cTnI was an independent predictor of hypertension, but not of ocular-TOD. With treatment of hypertension, cTnI did not decrease, which indicates that it is less useful for assessing the effect of treatment. The human medical literature currently is still confusing, with most studies reporting increases in cTnI in hypertensive patients,<sup>19,51,52</sup> but a recent study reported decreased cTnI concentrations in hypertensive HCM patients.<sup>53</sup> Increased SBP can lead to cardiac remodeling, with initial hypertrophy, followed by apoptotic loss of cardiomyocytes and fibrosis.<sup>54</sup> Hypertension may lead to some cardiomyocyte damage and necrosis, but the majority of cells may not have a severely damaged sarcolemma, and therefore, release of cTnI may occur in an alternative manner. Indeed, cell culture studies have confirmed that stimulation of integrins (mechanotransducing molecules in cardiomyocytes) leads to the release of intact cTnI,<sup>37</sup> and excised rat hearts put under increased pressure secrete cTnI products independent of ischemia.<sup>55</sup> Stretch of cardiomyocytes therefore may induce cTnI release, which may explain why, in our study, cTnI was identified as an independent predictor of hypertension. Despite cTnI being an independent predictor of hypertension, the hypertensive cats did not have significantly higher cTnI concentrations than did the CKD cats. Most hypertensive cats (36 of 49) had underlying CKD, and damage to the heart caused by CKD is a potential explanation for the higher concentrations found in all groups when compared to healthy cats. Additionally, effective antihypertensive treatment did not decrease plasma cTnI concentration. Cardiac troponin I has a long plasma half-life,<sup>16,56</sup> but hyperthyroid cats undergoing radioactive iodine treatment have decreased cTnI concentrations 3 months after treatment, indicating that the lack of a decrease at the 5-month time point in our study cannot be explained by the long half-life of cTnI.<sup>46,57</sup> This observation strengthens the hypothesis that cTnI is higher in all groups when compared to healthy cats because of underlying CKD. Because CKD is a risk factor for the development of hypertension,<sup>58</sup> the association between CKD and cTnI may have resulted in the predictive relationship between cTnI and hypertension.

The ultimate aim of identifying biomarkers would be to develop a diagnostic test that can help veterinarians in the challenging diagnosis of hypertension in the absence of ocular-TOD. When assessing the ROC curves (sensitivity and specificity), none of the biomarkers alone met the criteria to diagnose hypertension.<sup>59,60</sup> When combining all 3 potential markers with an AUC

that was significantly different from the line of zero discrimination, using a cutoff for VEGF of 76.1 pg/mL, NT-proBNP of 134.5 pmol/L, and cTnI of 0.045 ng/mL, the specificity can be increased to 96%. This is however associated with a sensitivity of 31%. A combination of cTnI and VEGF has a specificity 88% and sensitivity of 76% to diagnose hypertensive ocular-TOD. One cannot argue in favor of replacing a noninvasive method (retinal examination) with an invasive blood test with a lower sensitivity and specificity. This indicates that, although associated with blood pressure, hypertension, and ocular-TOD, these biomarkers are unlikely to be of clinical utility in the differentiation of white coat from true hypertension when no ocular-TOD is present.

In conclusion, plasma VEGF and cTnI concentrations are independent predictors of hypertension. Vascular endothelial growth factor may play a role in the development of hypertension or ocular-TOD associated with hypertension, and further research is warranted. Both plasma cTnI and VEGF concentrations may be indicative of less reversible tissue injury, and neither factor decreased with antihypertensive treatment. Plasma NT-proBNP concentration possibly could be used to assess efficacy of antihypertensive treatment. However, none of the biomarkers performed sufficiently well in predicting ocular-TOD to serve as a clinical test for hypertension or ocular-TOD.

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## Footnotes

- <sup>a</sup> Parks Electronic Doppler Model 811B, Perimed UK, Bury St Edmunds, UK
- <sup>b</sup> Mistral 3000, Sanyo-Gallenkamp, Leicestershire, UK
- <sup>c</sup> IDEXX Laboratories, Wetherby, Yorkshire, UK
- <sup>d</sup> Istin, Pfizer, Kent, UK
- <sup>e</sup> Canine VEGF Quantikine ELISA, R&D systems, Minneapolis, USA
- <sup>f</sup> GraphPad Prism version 6 for Windows, GraphPad Software, La Jolla, California, USA and R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

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*Conflict of Interest Declaration:* Authors declare no conflict of interest.

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

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