# The effects of illness on urinary catecholamines and their metabolites in dogs

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## ABSTRACT

**Background:** Urinary catecholamines and metanephrines have been proposed as a diagnostic tool for identifying canine pheochromocytomas, but the effects of critical illness on urine concentrations of catecholamines and metanephrines is currently unknown.

**Objectives:** To examine the effects of illness on urine concentrations of catecholamines and metanephrines in dogs.

**Animals:** Twenty-five critically ill dogs and twenty-five healthy age- and gender-matched control dogs.

**Methods:** Prospective observational study. Urine was collected from healthy and critically ill dogs and urine concentrations of epinephrine, norepinephrine, metanephrine, and normetanephrine were measured by high-performance liquid chromatography (HPLC) with electrochemical detection. Urinary catecholamine and metanephrine:creatinine ratios were calculated and compared between groups.

**Results:** Urinary epinephrine, norepinephrine, metanephrine, and normetanephrine:creatinine ratios were higher in critically ill dogs when compared to a healthy control population ($P = 0.0009$, $P < 0.0001$, $P < 0.0001$, and $P < 0.0001$ respectively).

**Conclusions and Clinical Relevance:** Illness has a significant impact on urinary catecholamines and their metabolites in dogs. Further investigation of catecholamine and metanephrine concentrations in dogs with pheochromocytomas is warranted to fully evaluate this test as a diagnostic tool, however the findings of this study suggest that the results may be difficult to interpret in dogs with concurrent illness.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACTH | adrenocorticotrophic hormone |
| ALP | alkaline phosphatase |
| ALT | alanine aminotransferase |
| ANOVA | analysis of variance |
| BID | *bis in die* (twice daily) |
| CBC | complete blood count |
| COMT | catechol-O-methyltransferase |
| CT | computed tomography |
| DHMA | 3,4-dihydroxymandelic acid |
| DHPG | 3,4-dihydroxyphenylglycol |
| DOPEGAL | 3,4-dihydroxyphenylglycoaldehyde |
| HCl | hydrochloric acid |
| HPLC | high performance liquid chromatography |
| HPLC-ED | high performance liquid chromatography with electrochemical detection |
| ICU | intensive care unit |
| LC-MS/MS | liquid chromatography with tandem mass spectrometry |
| MAO | monoamine oxidase |
| MIBG | metaiodobenzylguanidine |
| MHPG | 3-methoxy-4-hydroxyphenylglycol |
| MOPEGAL | 3-methoxy-4-hydroxyphenylglycoaldehyde |
| MRI | magnetic resonance imaging |

| | |
|---|---|
| PET | positron emission tomography |
| PNMT | phenylethanolamine N-methyltransferase |
| PO | *per os* (by mouth) |
| SID | once daily |
| SULT1A3 | sulfotransferase isoenzyme |
| TID | *ter in die* (three times daily) |
| VMA | vanillylmandelic acid |
| VMRCVM | Virginia-Maryland Regional College of Veterinary Medicine |

# INTRODUCTION

The catecholamines, epinephrine and norepinephrine, are synthesized within post-ganglionic sympathetic nerve fibers and chromaffin cells of the adrenal medulla.[1] They are released in response to activation of the sympathetic nervous system and act on α- and β-adrenergic receptors throughout the body to initiate a variety of physiologic responses to prepare the body for "fight or flight."[1-3] Pheochromocytomas are tumors that arise from the chromaffin cells and when functional, intermittently secrete catecholamines, causing a wide spectrum of clinical signs, including weakness, tachycardia, hypertension, and collapse.[4-8]

In humans, urinary catecholamines have been used for a number of years to diagnose pheochromocytomas. However, due to the paroxysmal nature of catecholamine secretion from the tumor, false negative results were common.[9-11] More recently, urinary and plasma metanephrines have come into favor as the screening test of choice for pheochromocytomas due to their increased sensitivity and specificity compared to catecholamines for pheochromocytoma diagnosis.[10-16] Within the chromaffin cells, there is constant metabolism of epinephrine and norepinephrine to the metanephrines, metanephrine and normetanephrine.[17,18] This metabolism occurs independently of catecholamine secretion and circulating epinephrine and norepinephrine are thought to be minor contributors to the production of metanephrines.[17,18] Therefore, urine and plasma concentrations of metanephrines are more consistently elevated, compared to catecholamines, in patients with pheochromocytomas.

Urinary metanephrines have been proposed as a diagnostic tool for dogs with pheochromocytomas, but the impact of stress and illness on these values has not been fully evaluated. There is some evidence that the stress of hospitalization can affect urinary metanephrines in healthy dogs,[19] and in humans, elevated metanephrine and normetanephrine concentrations have been observed in patients with ischemic strokes and concurrent infection.[20] As over fifty percent of dogs with pheochromocytomas have concurrent disease,[5,7,8] it is imperative to determine the effect of illness prior to investigating the diagnostic utility of urinary metanephrines for pheochromocytoma diagnosis in dogs.

The purpose of this study was to evaluate the effects of illness on urinary catecholamines and metanephrines in dogs. This will help assess the potential utility of this test for pheochromocytoma diagnosis in dogs in the future and will provide reference intervals to which patients with suspected pheochromocytomas can be compared.

# CHAPTER I: LITERATURE REVIEW

## A. The Adrenal Gland

### Anatomy

The adrenal glands are a pair of small triangular organs located cranial to the kidneys.[21] The glands are composed of an outer cortex and an inner medulla which form early in embryogenesis by migrating neural crest cells that become surrounded by mesoblastic cells.[21] There are three zones within the adrenal cortex, the zona glomerulosa, zona fasciculata, and zona reticularis which function to synthesize and secrete mineralocorticoids, glucocorticoids, and sex steroid hormones respectively.[22] The adrenal medulla is made up of chromaffin cells which synthesize, store, and secrete the sympathomimetic hormones known as catecholamines.[2,21] These cells act as specialized sympathetic ganglia in concert with the sympathetic nervous system to form the sympathoadrenal neuroendocrine system.[2] Preganglionic sympathetic neurons originating from the spinal cord synapse on chromaffin cells, causing the release of catecholamines into the bloodstream, which act on alpha and beta adrenergic receptors throughout the body to mediate the "fight or flight" physiologic responses to stress.[1,2]

### Catecholamine Synthesis

Catecholamines are synthesized in the axoplasm of post-ganglionic sympathetic nerve fibers as well as within the chromaffin cells of the adrenal medulla.[1] Tyrosine is taken up by catecholamine-secreting cells where it undergoes hydroxylation to dopa which is then decarboxylated to dopamine.[1,4,23] Dopamine is transported into secretory vesicles where it becomes hydroxylated into norepinephrine.[1,23] Within chromaffin cells, a large percentage of the norepinephrine is methylated to form epinephrine.[1] Phenylethanolamine N-methyltransferase (PNMT) in the cytoplasm performs this conversion on norepinephrine molecules that have leaked out of their vesicles.[23]

The rate-limiting step in catecholamine synthesis is tyrosine hydroxylation by tyrosine hydroxylase.[24] Norepinephrine within the cell cytoplasm acts to inhibit tyrosine hydroxylase activity and downregulates catecholamine production.[24] Sympathetic nervous system activation

results in release of intracellular norepinephrine, thus removing the negative feedback and allowing tyrosine hydroxylase activity to resume.[21]

Catecholamine Storage and Secretion

Catecholamines are stored in granules within the chromaffin cells and nerve terminals of post-gangliolic sympathetic nerves.[1,3] Preganglionic sympathetic nerve fibers synapse on post ganglionic neurons in the sympathetic chain ganglia or one of the peripheral sympathetic ganglia, releasing acetylcholine.[1,3] This stimulates the release of norepinephrine from the postganglionic nerve terminals.[1] Other preganglionic sympathetic nerve fibers travel directly to the adrenal medulla where they synapse and promote the release of epinephrine and norepinephrine from the chromaffin cells into the blood stream.[1,3]

Following their release, catecholamines act on α- and β-adrenergic receptors throughout the body and have a variety of far-reaching effects. Norepinephrine has a greater affinity for α-receptors while epinephrine excites both α- and β-receptors equally.[1] Sympathetic stimulation of the circulatory system leads to an increased heart rate and contractility as well as vasoconstriction of most systemic blood vessels, the consequence of which is increased blood pressure.[1,3] Peristalsis is inhibited in the gastrointestinal system and epinephrine stimulates gluconeogenesis within the liver.[1,3] Glycogenolysis in skeletal muscle and lipolysis in fat cells occur to accommodate a general increase in metabolism.[1,3] Bronchodilation, pupil mydriasis, and an increased strength of skeletal muscle contractions are all possible consequences of catecholamine release.[1,3]

Catecholamine Metabolism

Epinephrine and norepinephrine can be metabolized in two ways. In the first pathway, monoamine oxidase (MAO) deaminates norepinephrine or epinephrine to form 3,4-dihydroxyphenylglycoaldehyde (DOPEGAL).[17,25] Aldehyde reductase or aldose reductase then reduces DOPEGAL to 3,4-dihydroxyphenylglycol (DHPG).[26] DOPEGAL can also be metabolized by aldehyde dehydrogenase to form 3,4-dihydroxymandelic acid (DHMA), but due to the presence of a β-hydroxyl group on DOPEGAL, it is preferentially reduced by aldehyde or

aldose reductase.[17,27] DHPG diffuses out of the sympathetic nerves into the extracellular fluid where it is O-methylated by catechol-O-methyltransferase (COMT), found in extraneuronal tissues, to 3-methoxy-4-hydroxyphenylglycol (MHPG).[17,26] Alcohol dehydrogenase, located primarily in the liver, converts MHPG to 3-methoxy-4-hydroxyphenylglycolaldehyde (MOPEGAL)[28] which is then metabolized by aldehyde dehydrogenase to vanillylmandelic acid (VMA).[17,29] The liver also extracts catecholamines, metanephrines, and other metabolites from the bloodstream and converts them ultimately, by a similar mechanism, to VMA.[30]

In the second pathway, epinephrine and norepinephrine undergo O-methylation by COMT to form metanephrine and normetanephrine.[26] Further metabolism by MAO to MOPEGAL and reduction by aldehyde or aldose reductase to MHPG can also occur.[17]

Renal excretion is the primary route of elimination of catecholamines and their metabolites from the body.[23] Prior to renal elimination, the majority of these substances are first metabolized to sulfate conjugates.[23] Much of this sulfate conjugation takes places within the gastrointestinal tract, spleen, and pancreas where there are high concentrations of the sulfotransferase isoenzymes, SULTA1A3.[23,31] Clearance of the sulfate conjugates is slow as it is dependent on renal extraction, and their plasma half-lives are relatively long.[23] By comparison "free" (non-conjugated) catecholamines and metanephrines are extracted from the circulation very rapidly by tissues and organs throughout the body.[30] As a result, plasma concentrations of free catecholamines and metanephrines are much lower than their conjugated counterparts.[23]

The majority of catecholamine metabolism occurs within the same cells in which they are produced. There is a dynamic equilibrium between catecholamine transport into secretory vesicles and leakage back into the cytoplasm, where there is constant metabolism of the catecholamines.[17,18] Under resting conditions, the rate of leakage greatly exceeds the rate of exocytotic release.[17] As a result, the cellular uptake and metabolism of catecholamines by extraneuronal tissues after their release into circulation, accounts for less than 25% of total catecholamine metabolism.[17]

Sympathetic nerves do not contain COMT, thus all intraneuronal metabolism of norepinephrine is via MAO and leads to the production of DHPG.[17,18] Chromaffin cells of the adrenal medulla contain both COMT and MAO. However, COMT is in a membrane-bound form which has extremely high affinity for catecholamines.[17,23] As a result, the majority of intracellular catecholamine metabolism in chromaffin cells results in the production of metanephrine and normetaphrine.[17,23] Intracellular production of metanephrines in the adrenal glands accounts for 93% of circulating metanephrine and up to 40% of circulating normetanephrine, making the adrenal medulla the single largest source of both metanephrines in the body.[23,30] MAO does convert small amounts of catecholamines within the chromaffin cells to DHPG, but in comparison to the large quantity of DHPG produced by intraneuronal metabolism in sympathetic nerves, the amount produced by chromaffin cells is insignificant.[17] A soluble form of COMT is also found in other extraneuronal tissues such as smooth muscle cells and hepatocytes where it metabolizes small amounts of catecholamines extracted from the blood stream to metanephrines and converts DHPG to MHPG.[18]

The observation that metanephrine and normetanephrine synthesis occurs continuously and is independent of catecholamine secretion is what allows these metabolites to serve as sensitive markers for the presence of catecholamine-secreting tumors of the adrenal medulla, known as pheochromocytomas, in people. While catecholamine concentrations in patients with pheochromocytomas may fluctuate based on the paroxysmal secretory nature of the tumor, concentrations of metanephrines should remain steadily elevated. Over 94% of the plasma metanephrines in these patients are derived from the metabolism of catecholamines by COMT within the tumor cells, not from metabolism that occurs after epinephrine and norepinephrine are released into circulation.[32] Furthermore, given that the majority of metanephrine and normetanephrine production occurs intracellularly and total body concentrations are minimally influenced by extracellular metabolism of circulating catecholamines, other illness or stressors that may lead to catecholamine release would be less likely to affect concentrations of metanephrines.

Stress, Illness, and Catecholamines

Activation of the sympathetic nervous system and subsequent release of catecholamines can occur due to a number of physiologic and pathologic causes. Physiologic stimulation including quiet standing, isometric hand exercises, cold pressor tests, and vigorous exercise have all been shown to cause a significant increase in plasma concentrations of epinephrine and norepinephrine in humans when compared to resting values taken in a supine position.[33-35] Similarly, a study by Alexander, *et. al*. demonstrated that immobilization and noise stress caused a marked rise in plasma free catecholamines in rats.[36] Given that stress affects many dogs during routine examinations and clinic visits, it is important to understand the impact this might have on a diagnostic test. Urine collected at home from healthy dogs undergoing routine examinations and blood collection were found to have significantly higher urinary epinephrine and norepinephrine:creatinine ratios following a visit to the veterinarian when compared to levels measured 7 days later.[19] Their urine epinephrine and norepinephrine:creatinine ratios were also greater than those in dogs that were accustomed to the hospital environment and diagnostic procedures.[19]

Critical illness can lead to elevations in catecholamines as was observed by Woolf, *et. al.* in a study that demonstrated elevated free plasma norepinephrine and epinephrine in humans that sustained traumatic or vascular brain injury, polysystem trauma, or other illness requiring hospitalization in the intensive care unit (ICU).[37] Similar changes have also been seen in patients with liver failure, hypothyroidism, and myocardial infarction.[38] Although the effects of illness on canine catecholamine production are not well documented, unilateral nephrectomies on otherwise healthy dogs caused an intraoperative increase in the sum of plasma free epinephrine and norepinephrine.[39] Pituitary-dependent hyperadrenocorticism has also been shown to increase urinary epinephrine and norepinephrine:creatinine ratios in a small number of dogs as compared to a control group of dogs without adrenal disease.[40]

How these changes in catecholamine concentrations affect concentrations of metanephrines is less clear. Concentrations of metanephrines have been infrequently evaluated in humans with non-adrenal critical illness or those exposed to other potential mediators of sympathetic stimulation. Plasma free metanephrine and normetanephrine concentrations measured in humans the morning after an acute ischemic stroke were significantly elevated in

those that had concurrent infection as compared to those that did not.[20] Increased plasma normetanephrine concentrations at admission in these patients was also associated with increased 3 month mortality.[20] Kook's study found that in urine samples collected at home from healthy dogs undergoing routine examination and diagnostic testing at a veterinary clinic, urinary metanephrine and normetanephrine:creatinine ratios were not significantly different on the day of the exam (t0) as compared to 7 days before ( t-7) or 1 day after the visit (t1).[19] However, the metanephrine: creatinine ratio was significantly lower 1 week after the visit (t7) as compared to t-7, t0, and t1, and the normetanephrine:creatinine ratio was lower at t7 as compared to t-7.[19] Plasma free metanephrines were not significantly different in another study comparing dogs with blood collection performed at home versus those collected in the hospital.[41] In dogs with pituitary-dependent hyperadrenocorticism, urinary normetanephrine:creatinine ratios were higher than in normal dogs, but metanephrine:creatinine ratios were not different.[40] However, the effects of critical illness on concentrations of catecholamines and metanephrines in dogs have not been evaluated.

B. Adrenal Tumors

History and Incidence

With the increasing use of high-resolution diagnostic imaging in both human and veterinary medicine, there have been an increased number of patients diagnosed with incidentally discovered adrenal tumors. Clinically silent adrenal masses are detected in up to 5% of humans that undergo computed tomography (CT) or magnetic resonance imaging (MRI) for other reasons.[42-45] In veterinary medicine, abdominal ultrasound has allowed improved visualization of the adrenal glands, making the recognition of adrenal masses more common. Despite this, many adrenal tumors still go undiagnosed. A search of the Veterinary Medical Database over a 10-year period found a frequency of adrenal tumors among all canine patients of 0.17%.[46] However, necropsy studies of dogs in research colonies have yielded a much higher rate of occurrence: 10.8% of 2958 dogs that were necropsied had adrenal tumors.[46] Of the adrenal tumors diagnosed in these studies, 41-53% were adrenal cortical adenomas, 12-22% were adrenocortical adenocarcinomas, 12-16% were adrenomedullary tumors (including

pheochromocytomas, paragangliomas, and neuroepitheliomas), and 3-34% were metastatic lesions.[46]

Tumors of the adrenal gland are often functional. Hypersecretory tumors of the adrenal cortex produce excess cortisol, or occasionally other steroid hormones, leading to hyperadrenocorticism.[22,46-51] Functional tumors of the adrenal medulla, most commonly pheochromocytomas, secrete catecholamines causing stimulation of the sympathetic nervous system.[4-8,46,51-54] More rarely, pheochromocytomas in humans have been reported to secrete other peptides including somatostatin,[55,56] calcitonin,[57,58] vasoactive intestinal peptide,[59] corticotropin-releasing hormone,[60,61] growth hormone-releasing hormone,[62] adrenocorticotrophic hormone (ACTH), [63-66] parathyroid-like hormone,[67] and atrial natriuretic peptide[68] which can complicate their diagnosis.

The incidental discovery of an adrenal tumor can present a diagnostic dilemma to the clinician. Clinical signs may provide some clues as to whether an animal has hyperadrenocortism or catecholamine crises, but unfortunately, many times the clinical signs with these conditions are vague, intermittent, or non-specific. Additionally, there is overlap between the two as both dogs with pheochromocytomas and functional adrenocortical tumors may experience polyuria/polydypsia, weakness, and hypertension.[4-8,47,50] As many of these tumors are hypersecretory, biochemical testing may allow differentiation between adrenocortical and adrenomedullary tumors. An ACTH response test or low-dose dexamethasone suppression test may provide evidence of hyperadrenocorticism consistent with an adrenocortical adenoma or adenocarcinoma.[46,47,50] However, there are reports of dogs with both pheochromocytomas and adrenocortical tumors or pheochromocytomas and pituitary-dependent hyperadrenocorticism (a condition in which increased ACTH secretion from the pituitary gland leads to increased secretion of cortisol from the adrenal glands), which may confound the results. [7,69] Furthermore, the stress of another illness, such as a pheochromocytoma, might lead to increased adrenocortical activity, calling into question the results of these tests. ACTH secretion by a pheochromocytoma could lead to similar confusion.[63-66] Measuring plasma or urine concentrations of catecholamines and their metabolites are frequently employed in human medicine to diagnose

pheochromocytomas,[9-11,15,16,18,70-79] but their utility in veterinary medicine has not yet been thoroughly explored.[19]

Canine Pheochromocytomas

Pheochromocytomas are rare tumors in dogs that arise from the chromaffin cells in the adrenal medulla[7,8,46] or the sympathetic ganglia.[7,80] They are often functional, producing and secreting catecholamines, primarily epinephrine and norepinephrine, though some may produce dopamine or other polypeptide hormones. Although the majority of catecholamine secretion from normal adrenal medullary cells is in the form of epinephrine,[30] most human pheochromocytomas secrete primarily norepinephrine or a mixture of epinephrine and norepinephrine.[81] It is unknown if this occurs in dogs. The tumors occur in middle-aged to older dogs and have no gender or breed predisposition.[5-8,82] Clinical signs associated with pheochromocytomas are attributable to excessive secretion of catecholamines or local invasion of the tumor.[6-8] Diagnosis is often difficult due to the paroxysmal nature of catecholamine secretion and the nonspecific clinical signs associated with it. Though invasive tumors carry a guarded prognosis, successful surgical excision can be curative.[4,7,82,83]

Within pheochromocytoma cells, normal concentrations of intracellular norepinephrine fail to inhibit tyrosine hydroxylase, which normally acts as a rate-limiting step to regulate catecholamine synthesis.[24] This may be due to insensitivity of the negative feedback pathway to norepinephrine, because of sequestration of norepinephrine into cytoplasmic vesicles and its subsequent exocytosis, or because of intracellular metabolism of the catecholamine.[4,5] Because the tumor cells lack the innervation present in normal chromaffin cells, catecholamine release from the tumors is not neurally mediated.[5] The stimuli that do lead to catecholamine release in pheochromocytomas are poorly understood, though certain drugs,[84,85] physical pressure and manipulation, and changes in tumor blood flow may promote secretion.[3,5]

The majority of clinical signs associated with pheochromocytomas are attributable to the effects of catecholamine release or the space-occupying nature of the tumor and its metastases. Stimulation of $\alpha_1$-receptors leads to vasoconstriction and hypertension while activation of $\beta_1$-receptors can cause tachycardia and arrhythmias. There is evidence in rats that prolonged

periods of elevated catecholamines can lead to a down-regulation of $\alpha_1$- and $\beta_1$-receptors,[86,87] which may explain why some patients experience few to no clinical signs.

Pheochromocytomas have malignant tendencies in dogs. Local invasion has been identified in 33-52% of tumors[4,7,8] and metastasis in 13-36% of cases.[5,7,8] Spread to the regional lymph nodes, liver, spleen, lungs, and kidneys is most common, though metastasis to the brain, bone, heart, pancreas, spinal canal, and jejunum has also been reported.[7,8]

Clinical Findings

A variety of historical complaints and physical findings have been identified in dogs with pheochromocytomas, but the most common include weakness, lethargy, anorexia, weight loss, vomiting, polyuria/polydypsia, tachyarrthymias, tachypnea, pallor, and collapse.[4-8] Hypertension can lead to neurologic signs, including seizures and paresis, and hemorrhage in the retinas, or from nasal and gingival mucosa.[4-8] Large tumors can occasionally be palpated in the abdominal cavity and tumor invasion of the vena cava with subsequent thrombosis can lead to ascites or peripheral hindlimb edema.[4,5,7,8] Abdominal pain, restlessness, cough, fever, and rarely, cardiac arrest have also been reported.[5-8] Clinical signs are often episodic and some dogs may be completely asymptomatic. As a result, 48-60% of these tumors are diagnosed incidentally on ultrasound, exploratory celiotomy, or necropsy.[5,7,8]

Hematologic and biochemical findings are inconsistent and generally nonspecific. In one report, 64% of dogs were anemic, possibly due to chronic disease or blood loss from the tumor, while another found some dogs to be hemoconcentrated, which may be attributable to catecholamine-stimulated erythropoietin release or release of erythropoietin-like factors from the tumor.[4,5] Catecholamine release can cause decreased neutrophil margination resulting in neutrophilic leukocytosis, which has been found in 26-51% of dogs with pheochromocytomas.[7,8] Inflammation or necrosis of the tumor might also contribute to this leukocytosis. Increased liver enzyme activities, hypoalbuminemia, azotemia, and hypercholesterolemia are some of the more commonly reported biochemical abnormalities.[7,8] Increased alkaline phosphatase (ALP) and alanine aminotransferase (ALT) activities were found in 10% of dogs in one study[8] and 80% and 61%, respectively, in another,[7] but this has not been associated with hepatic metastasis.[5]

Twenty-two to 28% of dogs are hypercholesterolemic[7,8] which may reflect catecholamine-induced lipolysis and the liver's conversion of these fatty acids to cholesterol. However, concurrent disease could also explain a number of reported clinical pathologic abnormalities.

Because of the episodic nature of catecholamine secretion from pheochromocytomas, isolated blood pressure measurements are an insensitive diagnostic tool. Though it is a common finding in humans with pheochromocytomas,[88] only 38-50% of dogs with these tumors were hypertensive.[4,7] In one report, 9/10 hypertensive dogs with pheochromocytomas had concurrent diseases that could also cause an elevation in blood pressure.[7] Studies by both Gilson and Barthez determined that approximately 50% of the dogs with pheochromocytomas had concurrent, unrelated neoplasia.[7,8]

Diagnostic Imaging

Diagnostic imaging, although useful for identifying and evaluating the invasiveness of an adrenal mass, does not define the tumor's tissue of origin. Abdominal radiographs may identify a large mass in the retroperitoneal space, though this finding occurs in only 35-56% of dogs with pheochromocytomas.[4,5,8] Abdominal ultrasound is the most commonly used imaging technique to evaluate the adrenal glands in veterinary medicine.[89-93] Fifty to 85% of dogs with pheochromocytomas had adrenal masses identified on abdominal ultrasound, so although it is useful, some dogs with pheochromocytomas will go undetected despite imaging with ultrasound.[5,7] The typical ultrasonographic appearance is unilateral adrenomegaly with a normal-sized contralateral adrenal gland, although bilateral pheochromocytomas[7,8] and a dog with an adrenocortical tumor and a pheochromocytoma[69] have been reported. Many of the masses are cystic or multilobular and cause displacement of the kidney.[93] Ultrasound can also be useful for detecting metastatic disease or local invasion of the tumor into the caudal vena cava or other surrounding structures.[93] In one study, larger adrenal masses (> 2 cm) were more likely to be malignant, although there was no correlation between pheochromocytoma size and local invasiveness or with the severity of clinical signs.[90] In dogs with adrenal neoplasia, one report found ultrasound to have a sensitivity and specificity of 80% and 90% respectively, for detecting tumor thrombi in the vena cava.[94]

In humans with suspected pheochromocytomas and biochemical testing (catecholamines or metanephrines) consistent with the diagnosis, CT or MRI are the imaging modalities recommended for initial tumor confirmation and localization.[95] CT can detect masses larger than 5 mm as well as screen for metastatic lesions and local invasion. The sensitivity of CT for detection of a pheochromocytoma located within the adrenal gland in humans is between 77-98% with a specificity of 29-93% with the use of contrast enhancement.[95-97] The use of CT scans for the evaluation of adrenal disease has been limited in veterinary medicine. A report of CT scans of four dogs with pheochromocytomas found the modality to be more useful than radiographs or ultrasound for assessing the size, shape, and margination of the tumor and contrast enhancement aided in identification of surrounding vascular and urinary structures.[93] MRI is also a highly sensitive (90-100%) and specific (50-100%) technique for detecting pheochromocytomas in humans, and may have superior specificity for detecting extra-adrenal disease.[95-97]

After localizing an adrenal tumor, functional imaging studies are used in human medicine to confirm that a given mass is a pheochromocytoma and to exclude the presence of metastatic disease.[95] Metaiodobenzylguanidine (MIBG) is a guanethidine-derived analog that resembles norepinephrine and is taken up by sympathomedullary tissues. Using radioisotope labeled [$^{131}$I] or [$^{123}$I]MIBG for nuclear scintigraphy, areas with abnormally increased MIBG uptake can be identified. Since only adrenergic tissues will take up the radioisotope, it is a fairly sensitive (81%-90%) and very specific test (89%-100%).[96-100] For patients with elevated catecholamine or metanephrine concentrations but normal CT or MRI findings, a positive result can provide confirmation of a diagnosis.[98] Positron emission tomography (PET) with ligands such as 6-[$^{18}$F]fluorodopamine, [$^{18}$F]dihydroxyphenylalanine, [$^{11}$C]hydroxyephedrine, and [$^{11}$C]epinephrine, acts similarly, though it is not widely available.[95] The equipment required for functional imaging is not widely available in veterinary medicine and its use for pheochromocytoma diagnosis in dogs is limited to two case reports. [$^{123}$I]MIBG positively identified a tumor in a Yorkshire Terrier,[101] and the PET radiopharmaceutical p-[$^{18}$F]fluorobenzylguanidine, was used successfully to diagnose pheochromocytomas in 2 dogs.[102]

An adrenal mass was suspected in a third dog in the study, but PET imaging was negative and subsequent exploratory celiotomy confirmed the absence of a mass.[102]

C. Urinary and Plasma Catecholamines and Metanephrines

Use in Human Pheochromocytoma Diagnosis

Urine and plasma concentrations of catecholamines and metanephrines have been used in the routine screening of human patients for pheochromocytomas for a number of years. Recently, there has been a large body of literature published suggesting the superiority of measuring metanephrines versus catecholamines for pheochromocytoma screening and the consensus reached by the 2005 International Symposium on Pheochromocytoma states that initial testing for the tumor should always include measurements of either plasma free or urinary fractionated metanephrines.[12]

Plasma catecholamine and metanephrine concentrations offer the advantage of a brief blood draw as opposed to 24-hour urine collection that is recommended for sampling of urine catecholamines and metanephrines. Samples are drawn from patients in a sitting position or in a supine position after 30 minutes of rest. Lenders, *et. al.* found that the upper reference limits for plasma metanephrine and normetanephrine were higher in patients sampled in the sitting position than in those sampled in the supine position after rest. The group recommended patients be tested in a supine position or that testing be repeated in patients with high plasma metanephrines if blood was drawn while in the seated position.[103]

Multiple studies have evaluated the sensitivity and specificity of plasma metanephrines and catecholamines for pheochromocytoma diagnosis in humans. Sensitivities for plasma metanephrines ranged between 96-100% with specificities between 79-100%.[9,11,15,16,78,104,105] Metanalysis using data from five studies[13,15,16,78,105] calculated an overall diagnostic sensitivity of 98% and specificity of 92% for measurement of plasma free metanephrines.[106] Comparatively, the sensitivity of plasma catecholamines is reported from 70-85%[9,11,13,15,16,79] with specificities between 69-88%.[9,13,15,79]

Urinary metanephrines have traditionally been measured following acid hydrolysis or enzymatic deconjugation with sulfatase.[14] This step allows both the free and conjugated portions to be measured. Total urinary metanephrines represents a combined measure of the free and

conjugated portions of both metanephrine and normetanephrine while fractionated metanephrines delineates metanephrine (free + conjugated) from normetanephrine (free + conjugated). Free metanephrines contribute less than 3% to the total metanephrines in urine.[14] Because the gastrointestinal tract contains a high concentration of SULT1A3, it contributes a large and variable amount of conjugated normetanephrine to this pool, which may affect the diagnostic sensitivity and specificity of these tests.[14] Furthermore, conjugated metanephrines are cleared more slowly from the body than free metanephrines and are eliminated almost entirely by the kidneys.[14] Therefore, renal insufficiency can impact these measures. Unless otherwise specified, the measurement of urine catecholamines or metanephrines refers to the total of free and conjugated portions.

Studies comparing the diagnostic sensitivity and specificity of urine total or fractionated metanephrines with plasma free metanephrines documented sensitivities between 65-100%[9-11,13,15,78] and specificities from 69-95% for urinary metanephrines.[9,10,13,15,78] Of the studies that compared them directly, plasma free metanephrines were found to be a more sensitive test for pheochromocytomas than urinary metanephrines, but the specificity was not consistently greater. Similar to plasma catecholamines, urinary catecholamines have also proven to be a less consistent diagnostic test than its metabolized counterpart. The sensitivity of urinary catecholamines ranges from 74-93%[9-11,13,15,16,79] with specificity of 58.5-99%.[9,10,13,15,16,79] Recently, a study by Boyle, *et. al.*, evaluated the use of urinary free metanephrines for pheochromocytoma diagnosis, which allowed the less specific conjugated portion to be discounted. The sensitivity of the test in this study was 100% with 94% specificity, making it more sensitive than urinary catecholamines, urinary VMA, and plasma catecholamines to which it was compared. It was, however, less specific than urinary catecholamines or VMA.[79] Unfortunately, no comparison was made between plasma and urinary free metanephrines in this study, or in any others to date.

Urine for catecholamine and metanephrine analysis in humans is typically collected over a 24-hour period into a glass bottle containing concentrated hydrochloric acid. The acidification helps to maintain the stability of the compounds. Although this procedure is still most commonly followed, there have been studies evaluating the utility of single-voided samples for pheochromocytoma diagnosis. All of them found a strong correlation between concentrations of

metanephrines in the single voided samples compared to the 24-hour collection.[70,71] Metanephrine and normetanephrine concentrations were found to be consistent throughout the day[70] and when total metanephrine concentrations (metanephrine + normetanephrine) were assessed, single-voided samples had a high degree of specificity (100%) and sensitivity (97.6%) for detecting pheochromocytomas compared to a population of patients with non-functional adrenal tumors.[70]

Given that sensitivity is valued over specificity for pheochromocytoma diagnosis in humans, plasma metanephrines are gradually becoming the test of first choice for physicians. Because pheochromocytomas are rare tumors, this results in a high number of false positives. Urinary fractionated metanephrines has been suggested as a follow up test for patients with high plasma metanephrines.[12,107] Sequential testing with these two methods was shown in one study to increase the positive predictive value from 28.6% for plasma metanephrines alone to 86% when followed with urine fractionated metanephrines.[107]

Methodology

A number of different techniques for measuring urine and plasma catecholamines and metanephrines have been described. High performance liquid chromatography with electrochemical detection (HPLC-ECD) is the most often used method due to its high sensitivity and specificity.[108] Limitations of HPLC-ECD include extensive sample preparation, long analysis times and the potential for drug interference.[10,74,109] Recently, liquid chromatography with tandem mass spectrometry (LC-MS/MS) techniques have been developed which allow for more rapid turn-around time of samples and eliminates the problems caused by drugs due to analytical interference with HPLC-ECD methods.[110] This method has been validated in humans for the measurement of urinary and plasma catecholamines, urinary conjugated metanephrines, and plasma free metanephrines and reduces the run time of a single sample from 20 minutes with HPLC-ECD to 3-6 minutes using LC-MS/MS.[74,110-112] Furthermore, no interference from acetaminophen, chlorpromazine, desipramine, or ephedrine sulfate has been noted.[110] Comparison of plasma free metanephrine concentrations measured by LC-MS/MS and HPLC-

ECD in patients undergoing routine screening for pheochromocytomas showed a high degree of correlation, though LC-MS/MS is thought to have greater specificity.[74]

Use in Canine Pheochromocytoma Diagnosis

The evaluation of catecholamine and metanephrine concentrations for diagnosis of pheochromocytomas in veterinary medicine has been limited. Total plasma catecholamines were measured by Twedt and Wheeler in one dog diagnosed with a pheochromocytoma, and the values were elevated when compared to normal dogs.[4] Gilson, *et. al.* measured urine catecholamines and VMA in one dog with a pheochromocytoma, but found the results to be inconclusive.[8] Concentrations of urinary fractionated metanephrines in 2 dogs with pheochromocytomas were recently evaluated by Kook *et al*, who found elevated normetanephrine:creatinine ratios in both dogs. One of these dogs also had an elevated metanephrine:creatinine ratio.[19] Kook *et. al*. and Quante *et. al*., noted elevations in urinary normetanephrine:creatinine ratios in 6 dogs with pheochromocytomas,[40,113] but metanephrine and catecholamine:creatinine ratios overlapped those seen in healthy dogs.[113] Some overlap was also seen in normetanephrine:creatinine ratios between dogs with pheochromocytomas and those with pituitary-dependent hyperadrenocorticism.[40] The data in these reports are too sparse to conclusively determine the utility of metanephrines or catecholamines in the diagnosis of canine pheochromocytomas or the accuracy of plasma concentrations when compared to urine, particularly in animals with concurrent illness. At this time, establishing a diagnosis of a pheochromocytoma still requires histologic evaluation of a biopsy or excision of the mass.

Reference intervals in normal dogs need to be established before further use of these tests as diagnostic tools can be explored. Previous studies which have measured epinephrine and norepinephrine in dogs for other purposes have utilized small groups of normal dogs or compared post-treatment levels to baseline, but the actual values are often not reported or are only available in graphical form.[19,114-116] Unger, *et. al*. described a group of 10 female mixed-breed dogs with a mean baseline total plasma catecholamines of 0.71 +/- 0.1 ng/ml and total urinary catecholamines of 41.97 +/- 7.29 ng/ml.[117] Mean urinary epinephrine:creatinine ratios in 10 normal dogs were 3.9 +/- 1.3 nmol:mmol with norepinephrine:creatinine ratios of 1.5 +/- 0.3 nmol/mmol in Beerda *et. al*.'s study of hypoglycemia-induced stress.[118] An abstract by Kook, *et.*

*al*. reports mean urinary epinephrine and norepinephrine: creatinine ratios from 10 normal dogs as 4 nmol:mmol (range: 1-16) and 9 nmol:mmol (range: 3 – 67) respectively.[119] One of the largest studies evaluated 24 normal dogs and found mean plasma epinephrine concentrations of 1,370 +/- 730 pmol/L and norepinephrine concentrations of 3,420 +/- 1,220 pmol/L, but substantial individual variation was observed.[120] Similarly, another study of 12 dogs found mean plasma epinephrine concentrations of 2,222 +/- 27.3 pmol/L and mean norepinephrine plasma concentrations of 3,690 +/- 27 pmol/L.[121]

Concentrations of metanephrines in normal dogs have been evaluated far less frequently. In a conference proceeding presented by Dalessandri, *et. al*. plasma concentrations of free metanephrines were reported in 17 healthy dogs collected at home and 10 dogs collected in the clinic, with no significant differences noted.[41] Mean normetanephrine concentrations were 1,640.5 pmol/L +/- 864.9 and 1,875 pmol/L +/- 661.5 in dogs collected at home and in hospital, respectively, and mean plasma metanephrine concentrations were 1,603.5 pmol/L +/-680.5 in dogs collected at home versus 1,412 +/- 584.6 pmol/L in dogs collected at the hospital.[41] Mean urinary metanephrine and normetanephrine: creatinine ratios have been reported as 128 nmol:mmol and 66 nmol:mmol, respectively with values that ranged in 10 healthy dogs from 27-473 nmol:mmol (metanephrine:creatinine) and 14-103 nmol:mmol (normetanephrine:creatinine).[119]

## Factors Affecting Catecholamine and Metanephrine Concentrations

### *Renal Failure*

Urinary excretion is the primary route via which catecholamines and their metabolites are excreted from the body. Catecholamines and the free metanephrines are rapidly extracted from circulation by tissues within the body and further metabolized to sulfate conjugates or VMA.[23] The clearance of sulfate-conjugated metanephrines is dependent on renal extraction, resulting in slower clearance from the bloodstream when compared to their precursor metabolites.[23] Therefore, concentrations of sulfate-conjugated metanephrines are much higher than the concentrations of free metanephrines in plasma.[23] Because of their dependence on renal function, the plasma levels of sulfate conjugates can increase markedly in patients with renal failure.[122] Plasma levels of catecholamines and free metanephrines have been shown to be no

more than two-fold higher in humans with renal failure when compared with healthy volunteers, and they were increased above their respective reference ranges in less than 28% of the patients.[122,123] This increase is thought to be due to activation of the sympathetic nervous system that occurs in chronic kidney disease, rather than decreased renal clearance of the compounds.[122,124] Conversely, plasma concentrations of sulfate-conjugated metanephrines were increased above the upper limits of their reference ranges in 72% of patients with compromised renal function. Furthermore, a strong inverse relationship between creatinine clearance and plasma concentrations of conjugated normetanephrine and metanephrine has been demonstrated.[122] This can pose a challenge in diagnosing pheochromocytomas in patients that have compromised renal function, but suggests that significant elevations in plasma free metanephrines are still strongly suspicious of pheochromocytomas even with concurrent renal disease.

*Drug Interference*

A number of drugs have been shown to increase concentrations of catecholamines and their metabolites. In a study by Eisenhofer, *et. al*., phenoxybenzamine and tricyclic antipressants caused 1.9-2.6 fold increases in plasma and urinary concentrations of norepinephrine and normetanephrine when compared to patients taking other drugs or no medications. This can lead to a large number of false positive results when testing patients for pheochromocytomas.[125] Other $\alpha_1$-adrenoceptor blocking agents including doxazosin, terazosin, and prazosin, increased urinary concentrations of norepinephrine, but did not affect plasma or urine concentrations of epinephrine or the metanephrines.[125] β-blocking drugs elevated plasma metanephrine as well as urinary epinephrine, norepinephrine, metanephrine, and normetanephrine.[125] In addition, all patients taking sympathomimetics, such as pseudoephedrine, had elevated urinary metanephrines.[125] The elevated concentrations of catecholamines and metanephrines in these patients was associated with the drugs' effects on the systemic release of catecholamines.[125]

Drugs can also interfere directly with biochemical assays, particularly when using HPLC. Acetominophen, labetalol, captopril, and α-methyldopa can each cause additional chromographic peaks that can co-elute, partially or completely with the analytes of interest, leading to misidentification or improper estimation of their concentrations.[10,33,126-128] New

methods of HPLC have been developed to try to minimize this interference[129] and alternative techniques, such as mass spectrometry and immunoassays that rely on structural recognition of the compounds may help avoid these pitfalls.[110,126]

D. Pheochromocytoma Treatment

The treatment of choice for pheochromocytomas is surgical excision, however, anesthesia and surgical manipulation of the tumor have considerable risk. In a study of six dogs undergoing surgical treatment for pheochromocytomas, five experienced anesthetic complications including variable heart rate, cardiac arrhythmias, and hyper- or hypotension.[83] Reported post-operative complications include cardiac arrhythmias and arrest, dyspnea, sepsis, pancreatitis, acute renal failure, hemorrhage from the surgical site, disseminated intravascular coagulopathy, vomiting, and unresponsive hyper- or hypotension.[7,69,82,83,94] Post-operative mortality rates vary: Barthez, *et. al*. reported 8/17 dogs died or were euthanized during or within 10 days of surgery,[7] but studies from Kyles, *et. al.* and Gilson, *et. al*. had better survival rates with only 2/11 and 1/6 dogs dying in the perioperative period, respectively.[83,94] However, pre-operative treatment with phenoxybenzamine was recently shown to increase survival in dogs undergoing adrenalectomies for pheochromocytomas.[82] Phenoxybenzamine is a non-selective adrenergic antagonist of $\alpha_1$ and $\alpha_2$ receptors.[130] Blockade of these receptors opposes the vasoconstriction induced by circulating epinephrine and norepinephrine and helps control blood pressure in the weeks prior to surgery.[131] In humans, it has also been shown to decrease the incidence of intraoperative spikes in blood pressure that can be associated with induction of anesthesia and tumor manipulation,[131,132] Although a significant difference in intraoperative blood pressure variability has not been observed in dogs pre-treated with phenoxybenzamine, Herrera, *et. al*., found that untreated dogs undergoing adrenalectomies had a greater risk of dying in the peri-operative period compared to dogs pretreated with phenoxybenzamine.[82] Thus, prior knowledge of tumor type and appropriate pre-operative management can significantly increase survival.

To reduce the risk of surgical complications, or as long-term medical therapy in dogs with inoperable disease, treatment with pheoxybenzamine is recommended for 2-4 weeks prior to surgery.[4,5,82] Low doses (0.2 mg/kg *per os* [PO], twice daily) are given initially and

gradually increased up to 1.5 mg/kg or until improvements in clinical signs and reduced blood pressure occur.[4,5] For dogs with arrhythmias or severe tachycardia, β-adrenergic antagonists may also be beneficial, though they should always be given along with an α-blocker to prevent unopposed $\alpha_1$-mediated vasoconstriction.[4,5,53] Propranolol at 0.15-0.5 mg/kg PO three times a day or atenolol (0.2-1.0 mg/kg PO once or twice daily) can be used.[4,5,53]

Choosing appropriate anesthetic agents is important. Atropine should be avoided, given its propensity for worsening tachycardia.[4] Premedication with phenothiazines, particularly acepromazine, are contraindicated as they can lead to severe hypotension.[4] As patients with pheochromocytomas are prone to arrhythmias, halothane and barbiturates should also be avoided due to their arrhythmogenic properties.[4] Since histamine may provoke catecholamine release from some pheochromocytomas, drugs that stimulate mast cell degranulation, such as morphine and thiopental could also be problematic.[133] Recommended anesthetic protocols include premedication with an opioid and glycopyrrolate, induction with propofol or a benzodiazapine and maintenance on isofluorane or sevofluorane.[4,5,52] Diagnostic tests, such as measurement of plasma and urine catecholamines and metanephrines, that provide pre-surgical knowledge of tumor type, can allow for optimized surgical and anesthetic preparedness, and ultimately, improved surgical outcome. For dogs without metastasis that survive the immediate perioperitive period, long term survival of three years or more has been reported.[7]

# CHAPTER II: THE EFFECTS OF ILLNESS ON URINARY CATECHOLAMINES AND THEIR METABOLITES IN DOGS

## A. Introduction

Pheochromocytomas are uncommon tumors of dogs that pose a diagnostic challenge. These tumors of the chromaffin cells develop within the adrenal medulla or sympathetic ganglia.[7,8,46,80] When functional, pheochromocytomas secrete excessive quantities of catecholamines, leading to a variety of non-specific, and often paroxysmal, clinical findings including hypertension, tachyarrhythmias, weakness, and collapse.[4-8,53] Prognosis for dogs with invasive tumors is guarded, but surgical excision can be curative.[4,7,54,83] Clinical findings, blood pressure monitoring, and diagnostic imaging can be used to increase suspicion of a pheochromocytoma, but a convenient and reliable method to confirm the diagnosis is lacking.

With the increasing availability of ultrasonography, incidental adrenal masses are frequently identified. In patients with non-specific or inapparent clinical signs, it is often uncertain as to how testing should proceed when an adrenal mass is discovered. Pheochromocytomas cannot be distinguished ultrasonographically from adrenocortical tumors and many of the clinical signs seen with pheochromocytomas and hyperadrenocorticism overlap.[4-8,47,50] Testing for both conditions would seem prudent in any dog with an adrenal mass. However, at this time, biopsy is the only reliable method for pheochromocytoma diagnosis. As manipulation of these tumors can lead to massive catecholamine release, such procedures are not without risk.[4,5,54]

In humans, urine and plasma concentrations of catecholamines (epinephrine and norepinephrine) and metanephrines (metanephrine and normetanephrine) have been used for the routine screening of patients for pheochromocytomas for a number of years. Within the chromaffin cells, there is constant intracellular metabolism of catecholamines to metanephrines, creating stable concentrations that are minimally influenced by extracellular metabolism of circulating catecholamines.[17,18] In contrast, circulating catecholamine concentrations may vary in response to the secretory pattern of the tumor or to any number of stressors in patients without pheochromocytomas.[8,14,17,18] Because of this variability in catecholamine concentrations, measurements of metanephrines are preferred as they have been found to be more sensitive and

specific tests for the diagnosis of pheochromocytomas.[9-11,13,15,16] Urinary catecholamines and metanephrines in humans have traditionally been measured in urine collected over a 24 hour period. However, studies evaluating the concentrations in a single-voided sample have shown a high degree of sensitivity and specificity in the diagnosis of pheochromocytomas.[70]

The use of catecholamine and metanephrine concentrations as a diagnostic tool in veterinary medicine has been limited by cost, availability, and the lack of reference intervals for dogs.[4,52-54] Furthermore, the effects of non-adrenal illness on catecholamine and metanephrine concentrations in dogs are largely unknown. Critical illness in humans has been shown to elevate plasma catecholamine and metanephrine concentrations,[20,37,38] and while similar studies have not been performed in dogs, there is evidence that the stress of hospitalization can increase urinary catecholamines in dogs.[19] Fifty to sixty percent of dogs diagnosed with pheochromocytomas have concurrent disease.[5,7,8] Thus, a better understanding of the magnitude of the effect of non-adrenal illness on these values is vital for interpretation of catecholamine and metanephrine concentrations in dogs.

The primary objective of this study was to examine the effects of illness on urine concentrations of catecholamines and metanephrines in dogs. A secondary objective was to provide reference values that may aid in the diagnosis of canine pheochromocytomas. We hypothesized that critically ill dogs would have significantly higher urinary catecholamine:creatinine ratios compared to the control population, but that metanephrine and normetanephrine:creatinine ratios would not be significantly different between the groups.

## B. Materials and Methods

### Animals

The study population for this prospective study consisted of 25 critically ill dogs (Group 2) from the VMRCVM Veterinary Teaching hospital's intensive care unit (ICU) and 25 healthy, age- and gender-matched control dogs (Group 1). The study was approved by the Virginia Tech Animal Care and Use Committee. All owners signed informed consent. All dogs were greater than five years of age and did not have a history or evidence of congestive heart failure, renal disease, or adrenal disease based on physical examination, a complete blood count (CBC), biochemistry profile, urinalysis, indirect blood pressure measurement, and abdominal

ultrasonography or pathologic evaluation of the adrenal glands. Dogs were excluded if they had undergone surgery in the past month or had been given medications within the past two weeks that were known to affect catecholamine concentrations or to interfere with the biochemical assay (including phenylpropanolamine, prazosin, sympathomimetics, β-blockers, tricyclic antidepressants, and acetominophen).[125-128] Critical illness was defined as disease of significant severity to warrant hospitalization in ICU. Dogs in Group 2 were categorized by the primary disease process contributing to their illness. Case outcome was defined as surviving if the dog was discharged from the hospital and non-surviving if the dog died or was euthanized while in the hospital. Dogs in Group 1 were determined to be healthy based on the lack of clinically important abnormalities identified on the physical examination and tests described above.

Experimental Protocol:

In all dogs, voided urine was collected to measure epinephrine, norepinephrine, metanephrine, and normetanephrine:creatinine ratios. In 8 dogs, 4 urine samples were collected over an 8-14 hour period to evaluate the consistency of urinary excretion of catecholamines and metanephrines throughout the day. This group consisted of 2 dogs from the critically ill population, 4 dogs from the healthy dog population, and 2 apparently healthy dogs which were excluded from the remainder of the study due to mild bilateral adrenomegaly.

Sample Collection and Processing

Ten ml of urine were placed into plain glass tubes containing 197 uL of 6 M hydrochloric acid (HCl), gently mixed, divided into aliquots in polypropylene tubes, and frozen at -70°C. Samples were processed and frozen within an hour of collection. Analysis of urinary catecholamines and metanephrines was performed within five weeks of sample collection.

Analysis of Urinary Catecholamines and Metanephrines

High performance liquid chromatography (HPLC) with electrochemical detection was used to measure urinary free epinephrine and norepinephrine concentrations and total (free plus conjugated) metanephrine and normetanephrine concentrations using commercial reagents[a,b] in accordance with previously reported methods.[19,134-136] All samples were run in duplicate. For

samples in which there was no recovery of an analyte, the measured value was recorded as the assay's lower limit of detection for that analyte.

The intrassay and interassay coefficients of variation were calculated using a pooled urine sample collected from healthy dogs. Aliquots of this sample were run 8 consecutive times on day 1 and duplicate samples were run 9 additional times over a period of 3 months. Stability of the frozen samples over time was assessed by comparing urine concentrations of catecholamines and metanephrines from fresh urine samples collected on day 1 with samples frozen for 3, 5, 8, 11, 12, 15, 25, 50, and 78 days at -70°C.

Urine creatinine concentrations were measured in unacidified urine using an automated analyzer on the day of collection.[c] Urine epinephrine, norepinephrine, metanephrine, and normetanephrine to urine creatinine ratios were calculated.

Statistical Analysis

Normal probability plots were generated to determine if continuous data followed a normal distribution. Subsequently, continuous data were summarized as medians if not normally distributed (epinephrine, norepinephrine, metanephrine, and normetanephrine:creatinine ratios), or means if normally distributed (age, body weight, average adrenal gland size, and blood pressure). Contingency tables were generated for categorical data (gender).

Body weight and age were compared between the groups using a 2-sample t-test while the numbers of male and female dogs were compared between the groups using a chi-square test. The stability of the compounds over a 78-day period was assessed using locally weighted robust scatter plot smoothing. Evaluation of the variability of epinephrine, norepinephrine, metanephrine, and normetanephrine:creatinine ratios within individual dogs over the course of a day was tested using mixed model analysis of variance (ANOVA) with dog as a random effect. Associations between age, body weight, blood pressure, and average adrenal gland size and the ratios (epinephrine, norepinephrine, metanephrine, or normetanephrine:creatinine ratios) were investigated using scatter plots followed by analysis of covariance. The effect of gender on each of the ratios was tested using the Wilcoxon rank sum test. For dogs in Group 2, the effect of case outcome on each of the ratios was tested using the exact Wilcoxon rank sum test.

Epinephrine, norepinephrine, metanephrine, and normetanephrine:creatinine ratios were compared among the 2 groups using Wilcoxon rank sum tests. Correlations between epinephrine

and metanephrine:creatinine ratios and between norepinephrine and normetanephrine: creatinine ratios were assessed using Spearman rank correlation coefficients. Reference intervals for the ratios in each of the groups were estimated using the non-parametric percentile method. Commercial software was used to perform all statistical analyses[d] and to calculate reference intervals.[e] Statistical significance was set to $P<0.05$.

C. Results

Study Population

The healthy group (Group 1) included 12 castrated male and 13 spayed female dogs. The median age was 9 years (range, 5-15) and the median body weight was 27.7 kg (range, 4-39 kg). Breeds represented included mixed breed (n=14), Labrador Retriever (n=4), Golden Retriever (n=2), Australian Cattle Dog (n=1), Australian Shepherd (n=1), Black and Tan Coonhound (n=1), Border Collie (n=1), and Norwich Terrier (n=1).

The critically ill group (Group 2) included 11 castrated males, 1 intact male, and 13 spayed females. The median age was 9 years (range, 5-13). Median body weight was 16.4 kg (range, 5-40.9 kg). Breeds included mixed breed (n=5), Labrador Retriever (n=3), Shih Tzu (n=3), Beagle (n=2), Dachshund (n=2), Shetland Sheepdog (n=2), Bernese Mountain Dog (n=1), Bichon Frise (n=1), Border Collie (n=1), Boxer (n=1), Cocker Spaniel (n=1), Pomeranian (n=1), Scottish Terrier (n=1), and Weimaraner (n=1). Diagnoses for dogs in Group 2 included immune-mediated disease (n=7), hepatobiliary disease (n=4), pancreatitis (n=3), gastrointestinal disease (n=3), neurologic disease (n=3), neoplasia (n=3), or respiratory disease (n=2). Eight of these dogs had multiple disease processes affecting two or more body systems but were classified according to the pathologic process that was considered to be the most life-threatening at the time of hospitalization. Twenty dogs in Group 2 survived until discharge from the hospital and five died or were euthanized. There were no differences between the groups in regard to gender ($P=1.0$) or age ($P=0.82$), although body weight was greater in Group 1 ($P=0.0086$).

All dogs had CBC and serum biochemistry profiles performed. Indirect blood pressure measurements were performed in all dogs, with the exception of one dog in Group 2 in which the measurement was inadvertently not recorded. This dog was eliminated from analyses evaluating the effects of blood pressure. One dog in Group 2 did not undergo abdominal ultrasound but was euthanized and had grossly and histopathologically normal adrenal glands at necropsy. The right

adrenal gland was not identified during ultrasound examination in a second dog in Group 2, but both adrenal glands appeared grossly normal during a subsequent exploratory laparotomy. Urine was collected from all dogs during natural voiding, with the exception of one dog in Group 1 in which a cystocentesis was performed to obtain an adequate sample.

Intrassay and interassay variability

The intrassay coefficients of variation were 9%, 4%, 7%, and 8% for epinephrine, norepinephrine, metanephrine, and normetanephrine, respectively. The interassay coefficients of variation were 31%, 11%, 13%, and 11% respectively for epinephrine, norepinephrine, metanephrine, and normetanephrine. One duplicate set of samples used to calculate the interassay coefficient of variation had epinephrine concentrations that were double the concentrations measured in all other samples. This pair of readings was considered an outlier, and when it was eliminated from analysis, the interassay coefficient of variation for epinephrine was 9.7%.

Stability of samples over time and variability within individual dogs

Locally weighted robust scatter plot smoothing for epinephrine, norepinephrine, metanephrine, and normetanephrine concentrations versus day showed that the compounds were stable over time (Figs 1-4). Urinary epinephrine, norepinephrine, metanephrine, and normetanephrine:creatinine ratios did not vary in individual dogs over an 8-14 hour period ($P$=0.071, $P$=0.15, $P$=0.77, and $P$=0.64 respectively) (Figs 5-8).

Urinary Catecholamines and Metanephrines

Urinary epinephrine:creatinine ratios in Group 2 (median, 6.0 nmol:mmol; range 1.1-33.2) were greater than in Group 1 (median, 2.12 nmol:mmol; range, 0.52-11) ($P$=0.0009) (Fig 9). Norepinephrine:creatinine ratios in Group 2 (median, 16.3 nmol:mmol; range, 5.9-131) were also higher compared to Group 1 (median, 3.46 nmol:mmol; range, 0.6-12) ($P$<0.0001) (Fig 10). Urinary metanephrine:creatinine ratios in Group 2 (median, 56.9 nmol:mmol; range, 20.6-208) were higher than in Group 1 (median, 23.7 nmol:mmol; range, 7.24-78) ($P$<0.0001) (Fig 11) as were urinary normetanephrine:creatinine ratios (median, 145 nmol:mmol, range 58.1-1,040 in Group 2; median, 51.8 nmol:mmol; range, 22.5-183 in Group 1) ($P$<0.0001) (Fig 12).

Calculated reference intervals for catecholamine and metanephrines:creatinine ratios in healthy and critically ill dogs are presented in Table 1. Urinary epinephrine:creatinine ratios in 4/25 and norepinephrine:creatinine ratios in 16/25 critically ill dogs were higher than the reference intervals calculated for healthy dogs. Metanephrine:creatinine ratios in 9/25 and normetanephrine:creatinine ratios in 10/25 critically ill dogs were above the reference intervals calculated for healthy dogs. Among all samples, there was a positive correlation between urinary epinephrine:creatinine and metanephrine:creatinine ratios ($\rho$=0.83, $P$<0.0001) (Fig 13) and between norepinephrine:creatinine and normetanephrine:creatinine ratios ($\rho$=0.92, $P$<0.0001) (Fig 14).

No associations were found between age, body weight, blood pressure, or average adrenal gland size, as measured on ultrasound, and epinephrine, norepinephrine, metanephrine, or normetanephrine:creatinine ratios (Table 2). Female dogs in Group 2 had a median metanephrine:creatinine ratio of 68.9 nmol:mmol (range, 32.2-208) which was greater than the metanephrine:creatinine ratios of male dogs in that group (median 47.2 nmol:mmol; range, 20.6-116) ($P$=0.042). No other significant associations between gender and catecholamine or metanephrine:creatinine ratios were observed (Table 3). Dogs in Group 2 that died or were euthanized did not have significantly different catecholamine or metanephrine:creatinine ratios than dogs in Group 2 that survived ($P$=0.45, $P$=0.38, $P$=0.89, and $P$=0.28 for epinephrine, norepinephrine, metanephrine, and normetanephrine:creatinine ratios respectively) (Table 4).

D. Discussion

The results of this study show that critical illness has a significant impact on urine concentrations of catecholamines and metanephrines in dogs. Activation of the sympathetic nervous system and subsequent release of catecholamines can occur in response to a number of physiologic and pathologic stimuli. Therefore, the increased urinary catecholamine:creatinine ratios in critically ill dogs were anticipated. The effects of stress on urinary catecholamines have been investigated by Kook, *et al* who found that healthy dogs undergoing routine examinations and blood collection had significantly higher urinary epinephrine and norepinephrine:creatinine ratios on the day of examination compared to urine samples collected seven days later.[19] Similarly, in humans, elevated plasma concentrations of catecholamines have been associated

with illness in patients suffering from traumatic or vascular brain injury, polysystem trauma, liver failure, hypothyroidism, and myocardial infarction.[37,38]

While increased sympatho-adrenal activity is thought to minimally impact metanephrine concentrations,[137] this study found that urinary metanephrine and normetanephrine:creatinine ratios were significantly higher in critically ill dogs when compared to a healthy control population. Plasma, and subsequently urine, concentrations of metanephrines are dependent on both intracellular metabolism of catecholamines within the chromaffin cells of the adrenal medulla and catecholamine metabolism that occurs in tissues throughout the body following release of the catecholamines into circulation. The metabolism of circulating catecholamines accounts for only 16-19% of normetanephrine production and 6-10% of metanephrine production, thus increased release of epinephrine and norepinephrine from the adrenal medulla should have a relatively minor impact on total body production of metanephrines.[30,137] However, these percentages are derived from experimental studies in humans that involved infusions of catecholamines labeled with radiotracers and may not be representative of the physiologic processes that occur during critical illness.

There have been few studies evaluating metanephrine and normetanephrine concentrations in critically ill patients. Chamorro, *et al* found humans with acute ischemic stroke and concurrent infection had higher plasma free metanephrine and normetanephrine concentrations than stroke patients without infection.[20] In the veterinary literature, Kook, *et al* observed that even healthy dogs had lower urinary metanephrine and normetanephrine:creatinine ratios at the end of a study where owners had to collect voided urine samples compared to the start of the study when the dogs were reportedly stressed by the procedure.[19] The majority of studies that have documented the diagnostic utility of metanephrines are based on study populations of humans suspected of having pheochromocytomas, but this typically does not include patients that are critically ill. The tests are largely performed on an outpatient basis in people with a history of hypertension, suggestive clinical signs, an incidentally discovered adrenal mass, or a genetic predisposition for the neoplasm.[9,13,15,16,78,105] In a study which included hemodialysis and intensive care unit patients in the control group, 3/45 of the control patients had plasma free metanephrine or normetanephrine concentrations that were >50% above the reference interval.[138] This observation, as well as the studies by Kook[19] and Chamorro[20] are

more consistent with the findings of the present study in which critically ill dogs had increased metanephrine and normetanephrine:creatinine ratios. The supposition that the increased metanephrine concentrations were truly due to metabolism of increased quantities of circulating catecholamines is further supported by the very strong positive correlation observed between epinephrine and metanephrine:creatinine ratios as well as between norepinephrine and normetanephrine:creatinine ratios in the dogs in this study.

Measuring total metanephrine and normetanephrine concentrations rather than free metanephrines may also have contributed to the observed increase in concentrations of metanephrines in critically ill dogs. Total metanephrines include the free and conjugated forms of metanephrine and normetanephrine. Because there is a high concentration of the sulfotransferase isoenzyme, SULT1A3, within the gastrointestinal tract, it is theorized that while the majority of free metanephrine and normetanephrine are formed within the adrenal medulla, the majority of sulfate-conjugated normetanephrine originates from the mesenteric organs through the activity of SULT1A3.[23] As sulfate-conjugated metanephrines are typically 20-30-fold higher than free metanephrines,[137] the substantial contribution of sulfate-conjugated normetanephrine by the gastrointestinal tract may mask the otherwise stable concentration of free normetanephrine produced by the adrenal glands. This is thought to be one explanation for the improved specificity of measuring free plasma metanephrines compared to total urinary metanephrines in the diagnosis of pheochromocytomas in human patients.[13] It is possible that critically ill dogs have increased extra-neuronal production of normetanephrine within their gastrointestinal tract, but as very little epinephrine is produced outside of the adrenal glands, this would not explain the observed increase in metanephrine. Sulfate-conjugated metanephrines are eliminated by the kidneys and are cleared from circulation more slowly than free metanephrines.[23] Since patients with renal disease were excluded from this study, reduced renal function in Group 2 would not account for the differences seen between groups.

Until catecholamine and metanephrine concentrations in dogs with pheochromocytomas are fully evaluated, the clinical utility of our calculated reference intervals is limited. An abstract presented by Quante reported 6 dogs with pheochromocytomas which had a median normetanephrine:creatinine ratio of 445 nmol:mmol with a range of 157-6430 nmol:mmol.[40] Although the same assay was used in Quante's study as the study reported here, variation that

may exist between laboratories prevents us from making accurate comparisons. However, it is interesting to note that many of our critically ill dogs had normetanephrine:creatinine ratios within the range reported in dogs with pheochromocytomas. Similarly, in the same study by Quante, there was overlap in the range of normetanephrine:creatinine ratios between dogs with pheochromocytomas and dogs with pituitary-dependent hyperadrenocorticism.[40] Epinephrine, norepinephrine, and metanephrine:creatinine ratios were not significantly different between the two groups.[40] This suggests that urinary metanephrines may not be adequately specific to diagnose pheochromocytomas in dogs with concurrent illness. However, further evaluation of these values in dogs with confirmed pheochromocytomas may allow for the identification of a cut-off point above which a pheochromocytoma is likely even in the face of other underlying disease.

One weakness of this study was the use of only abdominal ultrasound and historical information to exclude the possibility of adrenal disease in the study population. Retrospective studies of canine pheochromocytomas indicate that only 50-85% of dogs with pheochromocytomas had adrenal masses identified on abdominal ultrasound,[5,7] so it is possible that a small tumor may have gone unnoticed. However, using the gold standard of histopathology to eliminate the possibility of adrenal disease was clearly impractical. Given the rarity of pheochromocytomas, it seems unlikely that occult adrenal neoplasms in the critically ill group would have contributed significantly to the observed outcome.

Groups 1 and 2 were significantly different with regard to body weight. While age and gender have been shown to affect urinary catecholamine concentrations in humans,[139] there is no reported effect of body weight. In this study, no correlation was found between body weight and epinephrine, norepinephrine, metanephrine, or normetanephrine:creatinine ratios. Thus, we feel it is unlikely that this difference had an impact on the results.

In humans, catecholamine and metanephrine concentrations are traditionally measured in urine collected over a 24-hour period. However, studies have shown a high degree of correlation between catecholamine and metanephrine concentrations in a single-voided and 24-hour collected samples, as well as a high degree of sensitivity and specificity of metanephrines in single-voided urine samples for the detection of pheochromocytomas.[70,73,140] The use of catecholamine:creatinine ratios in single-voided urine samples is a far more practical technique in veterinary medicine and has been used in previous canine studies evaluating urinary

catecholamines and metanephrines.[19] In the present study, multiple urine samples collected from 8 dogs over the course of the day showed little variation in urine concentrations of catecholamines and metanephrines. Though only a small group of dogs were evaluated, this further validates the use of single-voided samples for the evaluation of urinary catecholamines and metanephrines.

This study shows that illness has a significant impact on urinary catecholamines and their metabolites in dogs. Further investigation of catecholamine and metanephrine concentrations in dogs with pheochromocytomas will be needed to fully evaluate their utility as a diagnostic test for this neoplasm. However, our findings suggest that the results may be difficult to interpret in dogs with concurrent illness. As many dogs with pheochromocytomas present during a crisis or have an adrenal mass that is incidentally discovered during an imaging procedure to investigate another illness, urinary metanephrines may not be adequately specific to provide an immediate diagnosis. However, testing during a period of disease quiescence or after recovery from an unrelated illness could improve specificity. Furthermore, evaluation of plasma or urinary free metanephrines is warranted to determine if eliminating the effect of sulfate-conjugation might also improve the sensitive and specificity of metanephrines for pheochromocytoma diagnosis in dogs.

# CHAPTER III: CONCLUSIONS

This study demonstrates that critical illness has a significant impact on sympatho-adrenal activity in dogs. Not only were urinary catecholamine concentrations significantly higher in critically ill dogs, but metanephrine and normetanephrine concentrations were also markedly elevated compared to a population of healthy dogs. This was an unexpected, but important finding, as circulating catecholamines have previously been reported to have minimal impact on concentrations of metanephrines.[137]

One objective of this study was to evaluate the potential diagnostic utility of urinary catecholamines and metanephrines for pheochromocytoma diagnosis in dogs. Our findings illustrate the pitfalls of simply comparing urinary metanephrines of dogs with suspected pheochromocytomas to those of healthy dogs. Many of the critically ill patients had urinary catecholamine and metanephrine concentrations that were markedly above the reference interval calculated for healthy dogs, and taken at face value, could easily be interpreted as evidence of a pheochromocytoma. Further studies will be necessary to better evaluate concentrations of catecholamines and metanephrines in dogs with pheochromocytomas to determine if there is a cut-off point above which a diagnosis of pheochromocytoma is likely even in the face of concurrent illness. If not, collecting urine at a time when the dog is not otherwise ill or stressed may be beneficial.

Should future research validate the clinical utility of urinary catecholamine and metanephrine:creatinine ratios for the diagnosis of pheochromocytomas, this study shows that spot urine samples may provide a simple alternative to 24-hour urine collection and that the analytes remain stable over time when frozen. Therefore, urinary catecholamines and metanephrine concentrations could easily be made more widely available in a diagnostic laboratory setting.

FOOTNOTES

[a] Urinary Metanephrines by HPLC, BIO-RAD Laboratories, Hercules, CA

[b] Urinary Catecholamines by HPLC, BIO-RAD Laboratories, Hercules, CA

[c] Olympus AU400 Chemistry Analyzer, Beckman Coulter, Inc., Brea, CA

[d] SAS Version 9.2, SAS Institute Inc., Cary, NC

[e] Medcalc, Medcalc Software, Mariakerke, Belgium

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# APPENDIX A: PRELIMINARY STUDY

Sample collection for this study began in August, 2008. At that time, the intention was to measure catecholamine and metanephrine concentrations in plasma and urine using a HPLC assay developed by the Virginia-Maryland Regional College of Veterinary Medicine Toxicology Laboratory. Over a 3-month period, plasma and urine samples were collected from 7 critically ill and 5 healthy dogs. Additionally, urine and plasma samples from 5 dogs (3 healthy, 1 critically ill, and 1 dog with an adrenal mass) were run immediately after collection and again after being frozen for 15 and 30 days at -70°C as part of a stability study.

The lower limit of detection of the assays for catecholamines was $1x10^{-8}$ M and $5.5x10^{-5}$ M for metanephrines. Plasma concentrations of normetanephrine were undetectable in all 12 dogs. A measurable amount of plasma metanephrine was present in 1/12 dogs. Plasma concentrations of epinephrine were detectable in only 3/12 dogs and norepinephrine was detected in 9/12 dogs. Measurable concentrations of metanephrine and normetanephrine were present in urine from all dogs. Urinary epinephrine was detectable in 8/12 dogs and urinary norepinephrine was detected in 11/12 dogs.

For the stability study, plasma concentrations of norepinephrine were run 3 times in each of 3 dogs (Fig 15). Plasma concentrations of norepinephrine of the other 2 dogs in the stability study were undetectable on day 1, and further evaluation of the samples was not performed. The coefficients of variation for each of the 3 dogs were 43.6%, 15%, and 51.6%. Marked variability in the results was noted, but no consistent increase or decrease in the concentrations was observed. None of the five dogs had detectable levels of epinephrine, metanephrine, or normetanephrine on day 1, so the stability of these compounds when frozen was not performed.

Dogs that had undetectable urinary concentrations of epinephrine, norepinephrine, metanephrine, or normetanephrine on day 1 were eliminated from the stability study. Stability of the urine concentrations of epinephrine was assessed in 3 dogs which had coefficients of variation of 81%, 151%, and 105%. Coefficients of variation of urinary norepinephrine in 5 dogs were 89.8%, 45.4%, 25.6%, 93.4%, and 130%. Urinary metanephrines in 5 dogs had coefficients of variation of 103%, 48.6%, 73.8%, 118%, and 111%, and urinary normetanephrine coefficients of variation were 107%, 44%, 93%, 95.9%, and 117%. Because the plasma and urinary catecholamine and metanephrine concentrations were inconsistent over time rather than

showing a steady increase or decrease (Figs 15-19), the problem of interassay variability was thought to be due to an inherent problem with the HPLC assay rather than instability of the compounds themselves.

Given the poor recovery of the analytes in plasma and the unacceptable levels of interassay variability, alternative techniques for measuring catecholamines and metanephrines were explored. Attempts were made to develop an assay using liquid chromatography with tandem mass spectrometry, but this too had problems with poor recovery of analytes and high interassay variability.

Ultimately, commercially available HPLC columns and analytes were purchased.[a,b] These have been used in previous studies of urinary catecholamines and metanephrines in dogs.[19] Because catecholamines and metanephrines appear to be present in extremely low concentrations in canine plasma, a large volume of plasma would have been needed to run the assay. Collection of such a large volume of blood was felt to be impractical and unethical in our critically ill dogs and given the uncertainty that we would even be successful, further attempts at measuring plasma concentrations of catecholamines and metanephrines were not made.

For urinary catecholamines and metanephrine, the intrassay and interassay coefficients of variation were calculated using a pooled urine sample collected from healthy dogs. Aliquots of this sample were run 8 consecutive times on day 1 and duplicate samples were run 9 additional times over a period of 3 months. Stability of the frozen samples over time was assessed by comparing urine concentrations of catecholamines and metanephrines from fresh urine samples collected on day 1 with samples frozen for 3, 5, 8, 11, 12, 15, 25, 50, and 78 days at -70°C. The intrassay coefficients of variation were 9%, 4%, 7%, and 8% for epinephrine, norepinephrine, metanephrine, and normetanephrine, respectively. The interassay coefficients of variation were 9.7%, 11%, 13%, and 11% respectively for epinephrine, norepinephrine, metanephrine, and normetanephrine. The improved consistency and reliability of this assay allowed us to complete the project as described above.

APPENDIX B: FIGURES

**Figure 1.** Locally weighted robust scatter plot for epinephrine (EPI) concentrations in pooled urine samples frozen for up to 78 day at -70°C. Epinephrine concentrations were stable over time. Selected smoothing parameter: 0.396.

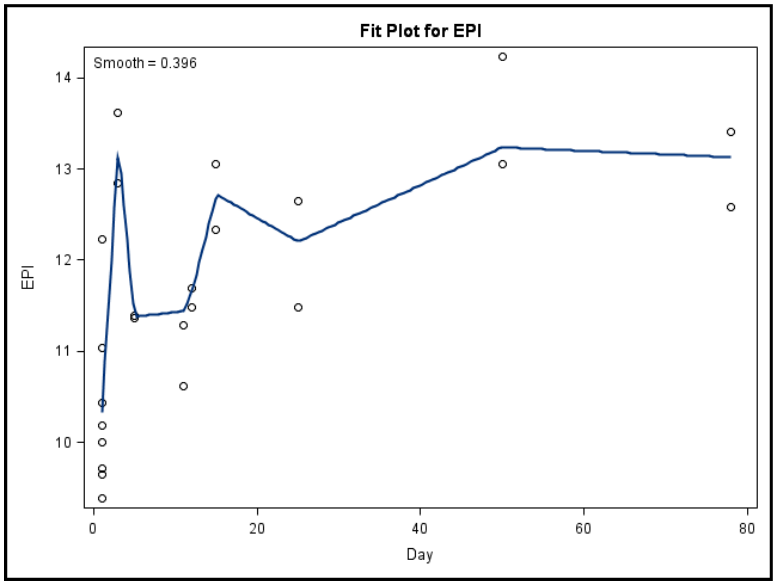



**Figure 2.** Locally weighted robust scatter plot for norepinephrine (NE) concentrations in pooled Urine samples frozen for up to 78 day at -70°C. Norepinephrine concentrations were stable over time. Selected smoothing parameter: 0.442.

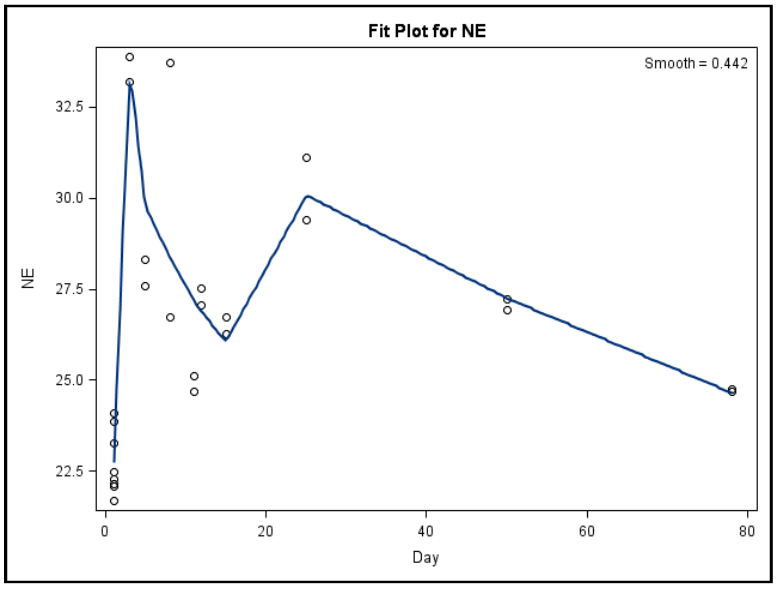


**Figure 3.** Locally weighted robust scatter plot for metanephrine (MN) concentrations in pooled Urine samples frozen for up to 78 day at -70°C. Metanephrine concentrations were stable over time. Selected smoothing parameter: 0.563.

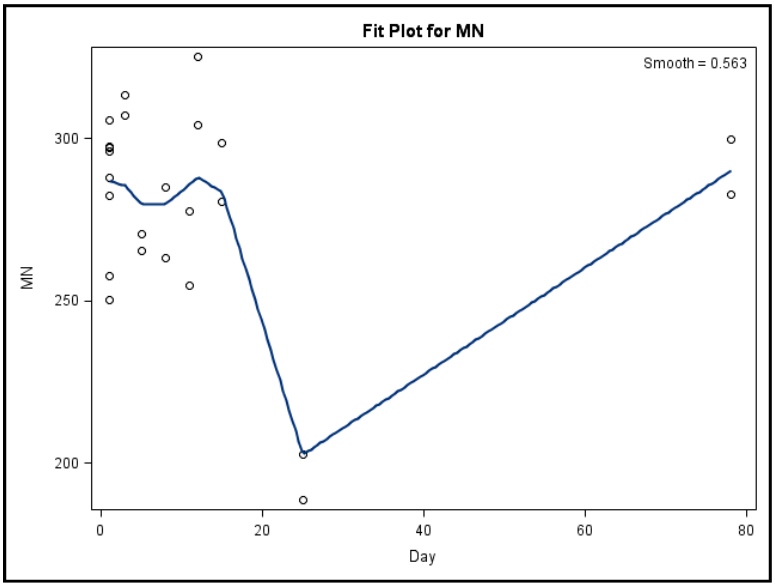



**Figure 4.** Locally weighted robust scatter plot for normetanephrine (NMN) concentrations in pooled urine samples frozen for up to 78 day at -70°C. Normetanephrine concentrations were stable over time. Selected smoothing parameter: 0.896.

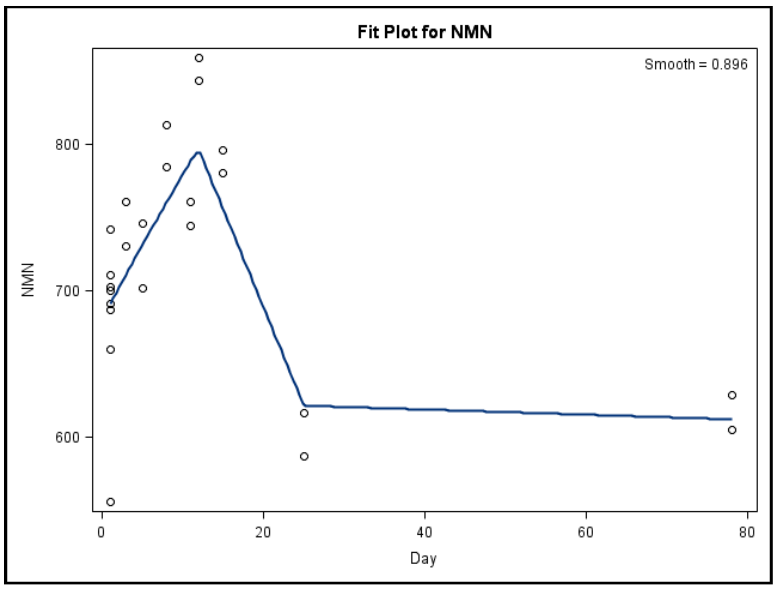


**Figure 5.** Variability of urinary epinephrine (EPI):creatinine ratios in 8 dogs measured 4 times over the course of a day. The ratios did not vary significantly in individual dogs over time (*P*=0.071)

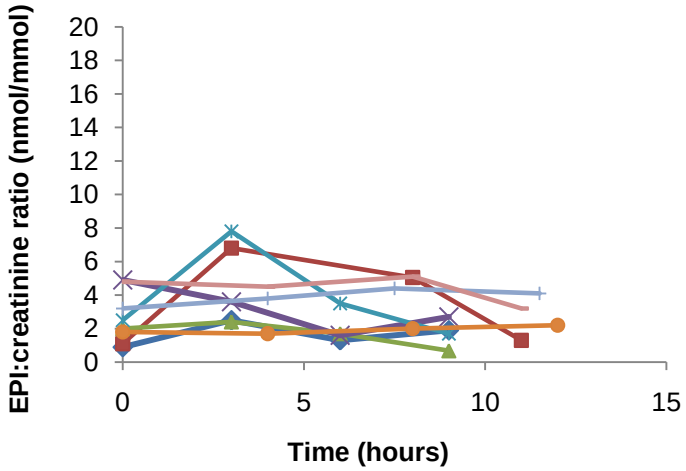



**Figure 6.** Variability of urinary norepinephrine (NE):creatinine ratios in 8 dogs measured 4 times over the course of a day. The ratios did not vary significantly in individual dogs over time (*P*=0.15)

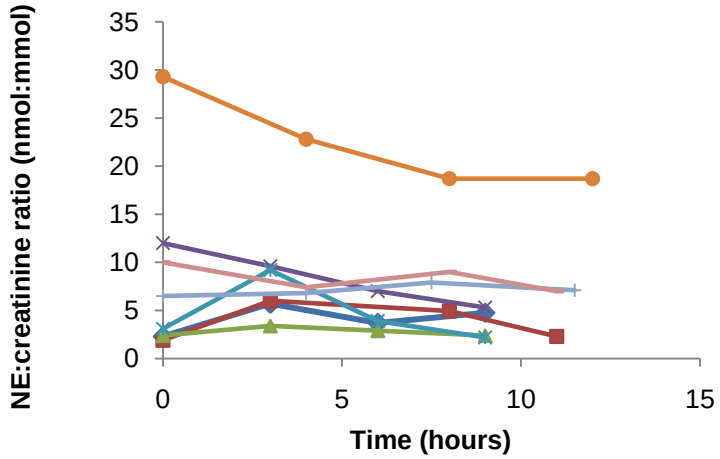


**Figure 7.** Variability of urinary metanephrine (MN):creatinine ratios in 8 dogs measured 4 times over the course of a day. The ratios did not vary significantly in individual dogs over time (*P*=0.77)

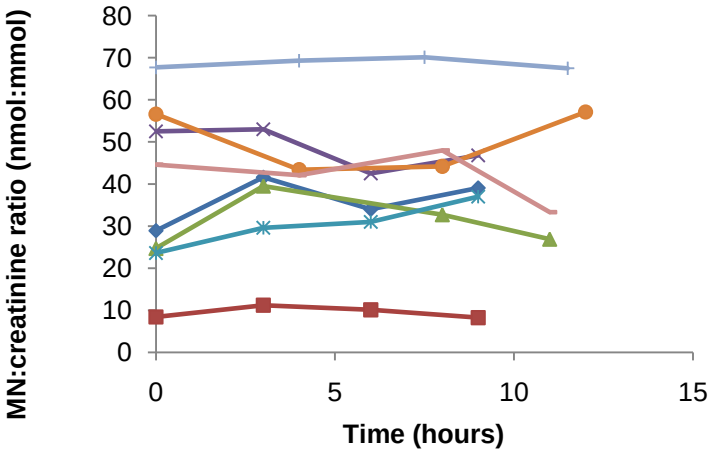



**Figure 8.** Variability of urinary normetanephrine (NMN):creatinine ratios in 8 dogs measured 4 times over the course of a day. The ratios did not vary significantly in individual dogs over time (*P*=0.64)

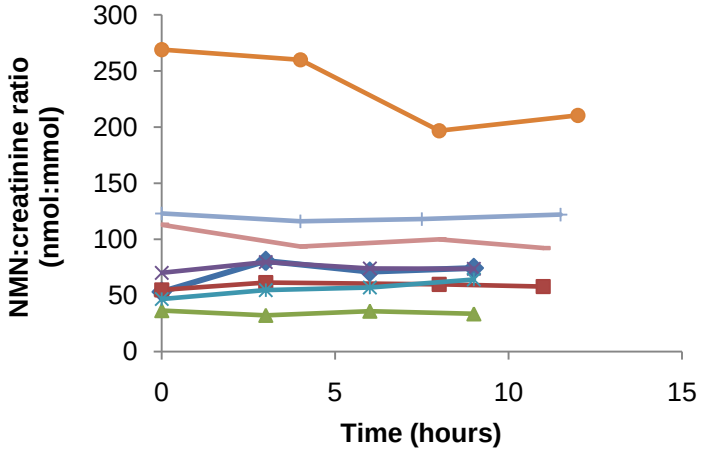
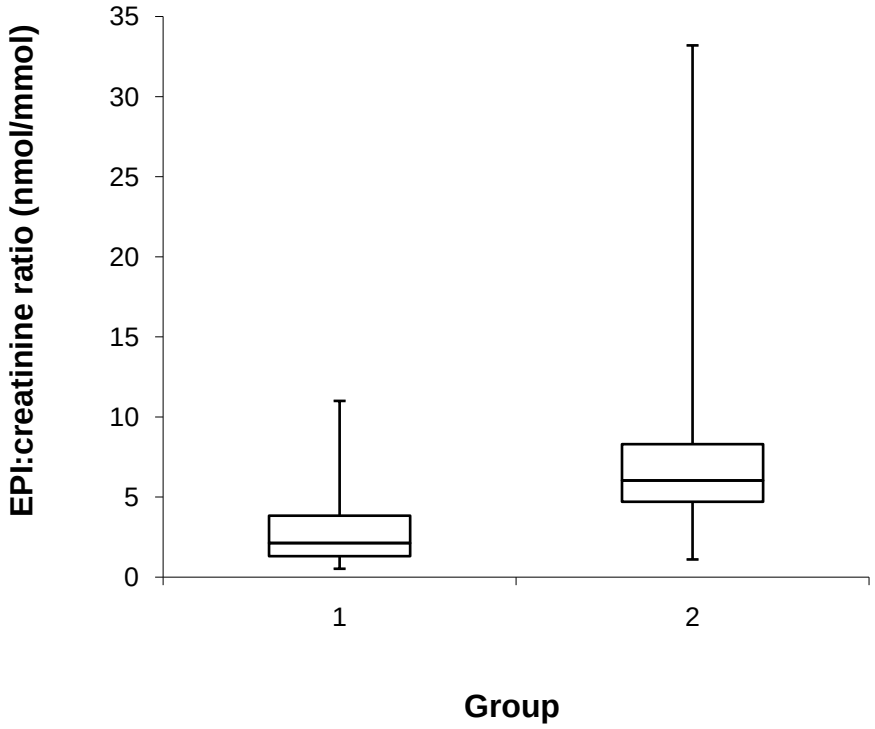
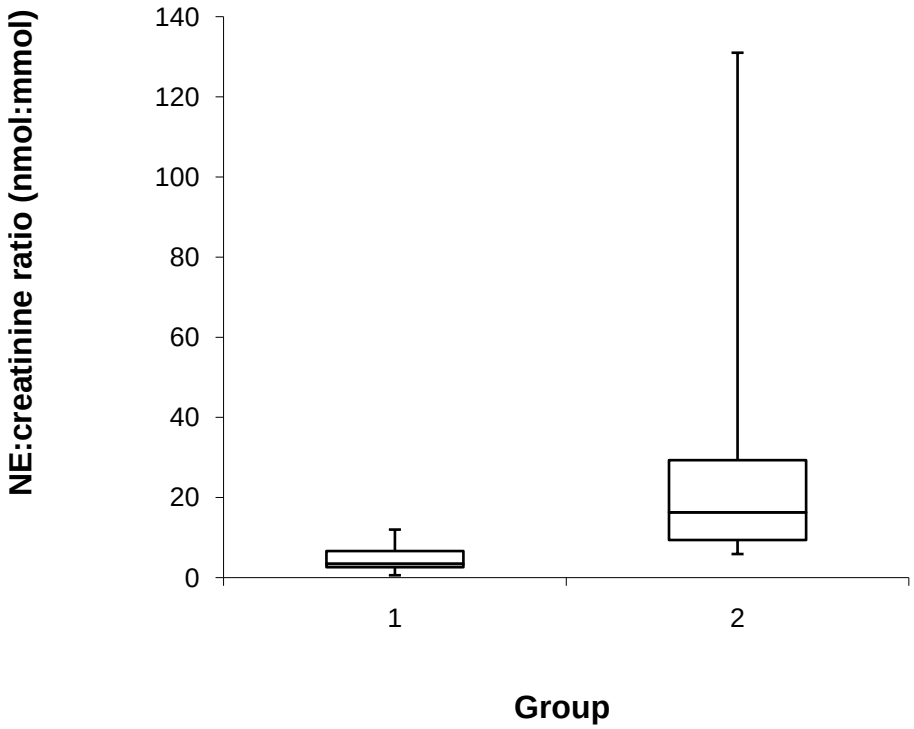


**Figure 9.** Comparison of urinary epinephrine (EPI):creatinine ratios between healthy dogs (Group 1, n=25) and critically ill dogs (Group 2, n=25). The ratios differed significantly between groups (*P*=0.0009).



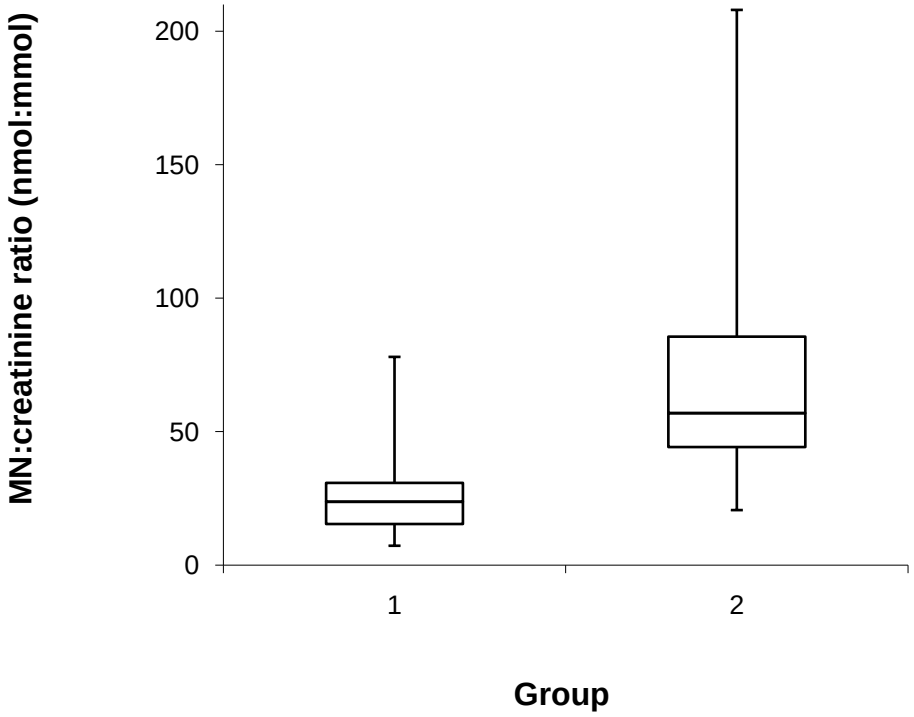

Box and whiskers plot - the boxes represent the interquartile ranges, the horizontal bars within the boxes indicate the medians, and the whiskers show the range.

**Figure 10**. Comparison of urinary norepinephrine (NE):creatinine ratios between healthy dogs (Group 1, n=25) and critically ill dogs (Group 2, n=25). The ratios differed significantly between groups ($P < 0.0001$).



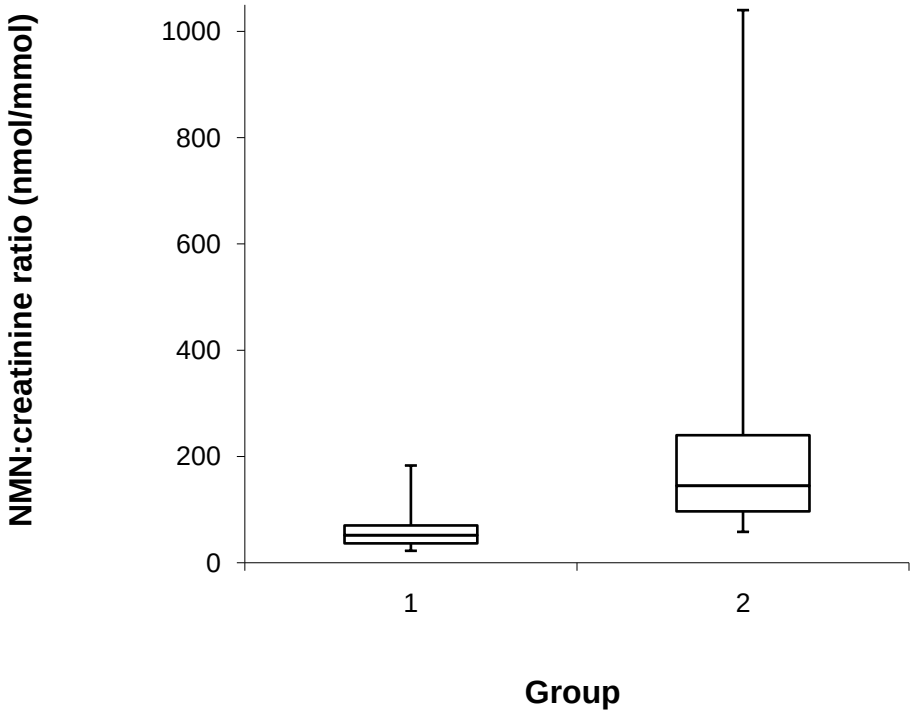

Box and whiskers plot - the boxes represent the interquartile ranges, the horizontal bars within the boxes indicate the medians, and the whiskers show the range.

**Figure 11**. Comparison of urinary metanephrine (MN):creatinine ratios between healthy dogs (Group 1, n=25) and critically ill dogs (Group 2, n=25). The ratios differed significantly between groups ($P < 0.0001$).




Box and whiskers plot - the boxes represent the interquartile ranges, the horizontal bars within the boxes indicate the medians, and the whiskers show the range.

**Figure 12.** Comparison of urinary normetanephrine (NMN):creatinine ratios between healthy dogs (Group 1, n=25) and critically ill dogs (Group 2, n=25). The ratios differed significantly between groups ($P < 0.0001$).




Box and whiskers plot - the boxes represent the interquartile ranges, the horizontal bars within the boxes indicate the medians, and the whiskers show the range.

**Figure 13.** Correlation between urinary epinephrine(EPI):creatinine ratios and metanephrine(MN):creatinine ratios among all dogs in the study. A significant positive correlation was observed (ρ=0.83, *P*<0.0001).

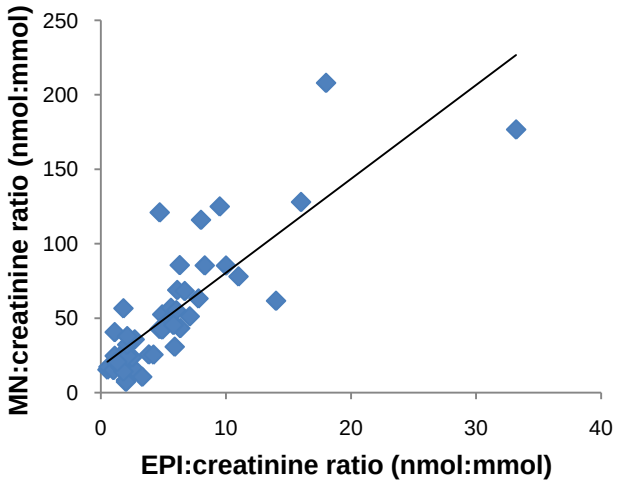



**Figure 14.** Correlation between urinary norepinephrine(NE):creatinine ratios and normetanephrine(NMN):creatinine ratios among all dogs in the study. A significant positive correlation was observed (ρ=0.92, *P*<0.0001).

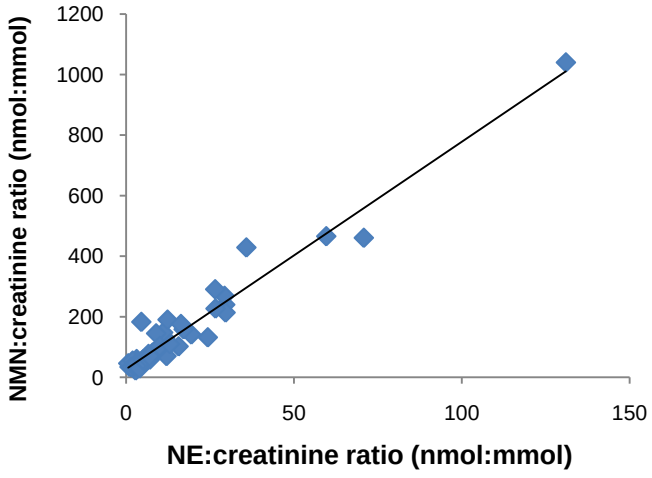


**Figure 15.** Plasma norepinephrine (NE) concentrations in 3 dogs run at the time of collection (day 0) and after being frozen for 15 and 30 days at -70° using initial VMRCVM assay that failed.

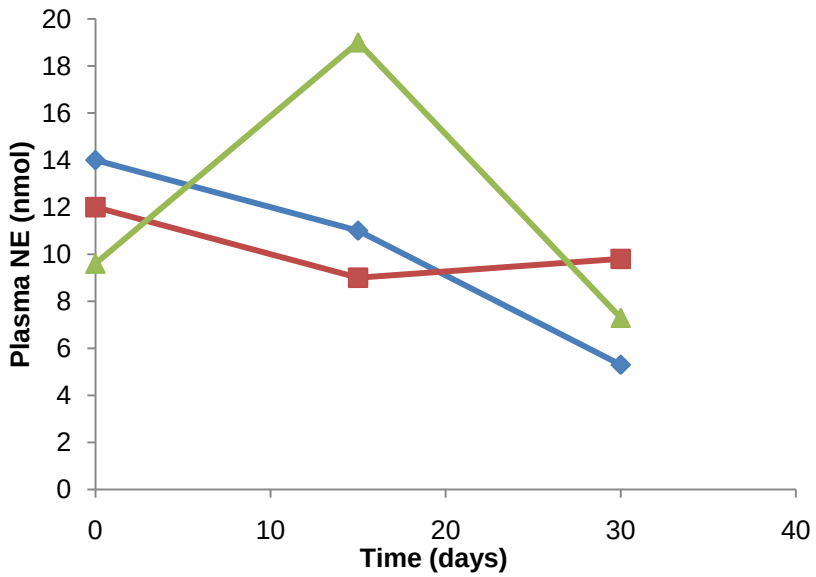


**Figure 16.** Urine epinephrine (EPI) concentrations in 5 dogs run at the time of collection (day 0) and after being frozen for 15 and 30 days at -70° using initial VMRCVM assay that failed.

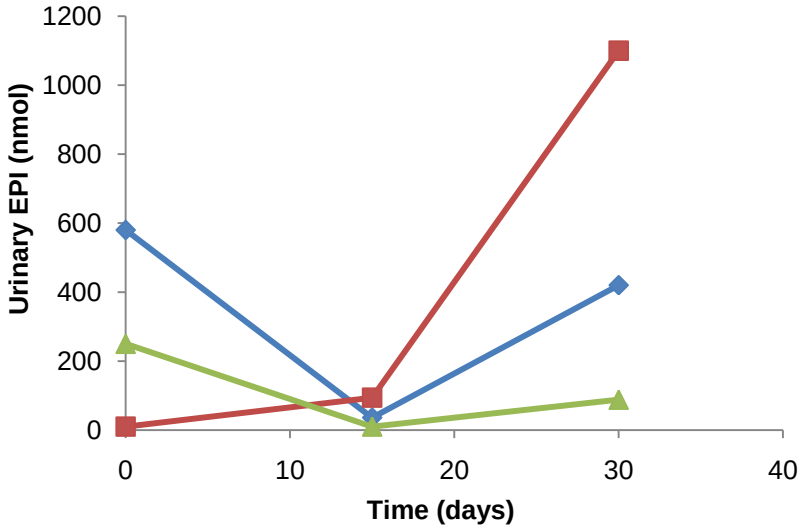



**Figure 17.** Urine norepinephrine (NE) concentrations in 5 dogs run at the time of collection (day 0) and after being frozen for 15 and 30 days at -70° using initial VMRCVM assay that failed.

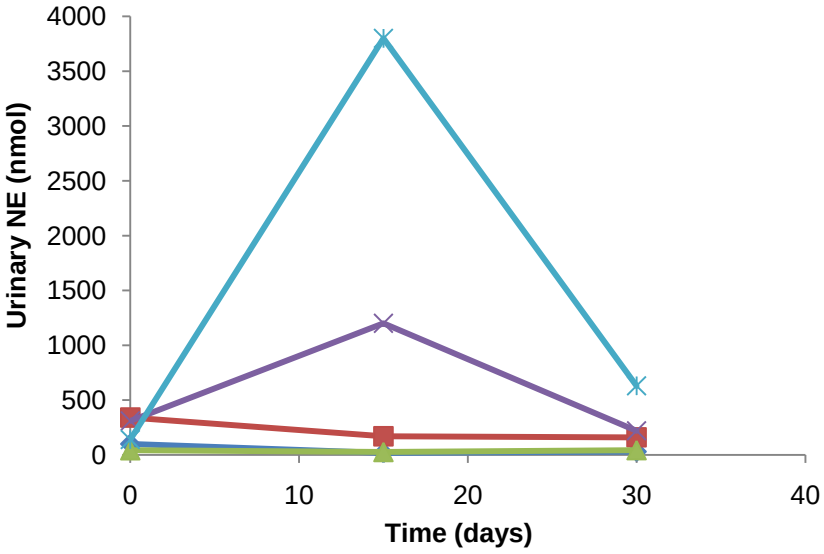


**Figure 18.** Urine metanephrine (MN) concentrations in 5 dogs run at the time of collection (day 0) and after being frozen for 15 and 30 days at -70° using initial VMRCVM assay that failed.

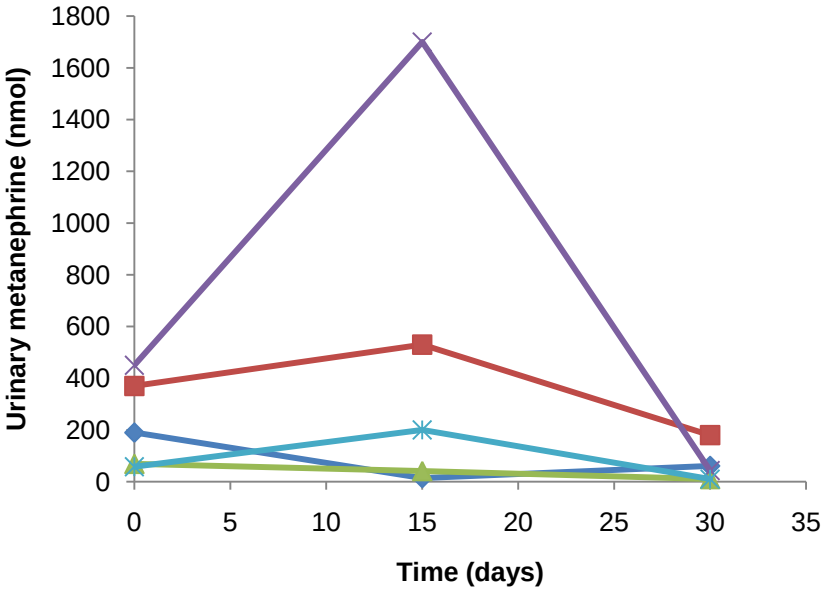
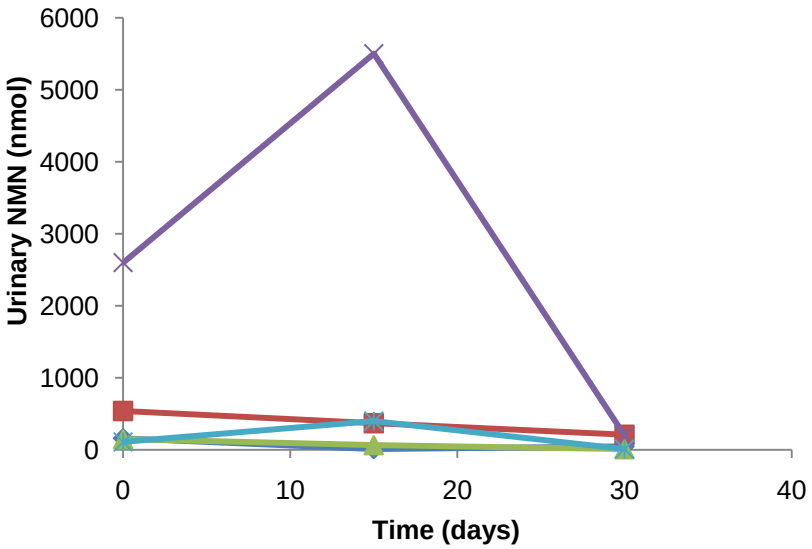



**Figure 19.** Urine normetanephrine (NMN) concentrations in 5 dogs run at the time of collection (day 0) and after being frozen for 15 and 30 days at -70° using initial VMRCVM assay that failed.



## APPENDIX C: TABLES

**Table 1.** Calculated reference intervals for urinary catecholamine and metanephrine:creatinine ratios in healthy and critically ill dogs

| | Healthy dogs | Critically ill dogs |
|---|---|---|
| EPI:creatinine (nmol/mmol) | 0.53-10.5 | 1.1-30.8 |
| NE:creatinine (nmol/mmol) | 0.64-11.9 | 6.0-121 |
| MN:creatinine (nmol/mmol) | 7.38-75.9 | 21.8-204 |
| NMN:creatinine (nmol/mmol) | 23.2-178 | 58.4-941 |

EPI = epinephrine; NE = norephinephrine; MN = metanephrine; NMN = normetanephrine.

**Table 2.** Associations between age, average adrenal gland size, blood pressure, body weight and catecholamine and metanephrine:creatinine ratios

| **Comparison** | | ***P* value** |
|---|---|---|
| Age | EPI:creatinine ratio | 0.071 |
| Age | NE:creatinine ratio | 0.43 |
| Age | MN:creatinine ratio | 0.18 |
| Age | NMN:creatinine ratio | 0.58 |
| Adrenal size | EPI:creatinine ratio | 0.86 |
| Adrenal size | NE:creatinine ratio | 0.84 |
| Adrenal size | MN:creatinine ratio | 0.99 |
| Adrenal size | NMN:creatinine ratio | 0.86 |
| Blood pressure | EPI:creatinine ratio | 0.14 |
| Blood pressure | NE:creatinine ratio | 0.96 |
| Blood pressure | MN:creatinine ratio | 0.07 |
| Blood pressure | NMN:creatinine ratio | 0.51 |
| Body weight | EPI:creatinine ratio | 0.87 |
| Body weight | NE:creatinine ratio | 0.68 |
| Body weight | MN:creatinine ratio | 0.12 |
| Body weight | NMN:creatinine ratio | 0.56 |

EPI = epinephrine; NE = norepinephrine; MN = metanephrine; NMN = normetanephrine

**Table 3.** Associations between gender and catecholamine and metanephrine:creatinine ratios within groups and among all dogs in the study.

| **Association with gender** | | **Group 1** | | **Group 2** | | **All dogs** | |
|---|---|---|---|---|---|---|---|
| | **Gender** | **M** | **F** | **M** | **F** | **M** | **F** |
| | *N* | 12 | 13 | 12 | 13 | 24 | 26 |
| EPI:creatinine ratio (nmol:mmol) | Mean | 3.0 | 3.1 | 5.1 | 10.2 | 4.0 | 6.6 |
| | SD | 2.7 | 2.4 | 2.4 | 8.6 | 2.8 | 7.2 |
| | *P* | 0.98 | | 0.13 | | 0.34 | |
| NE:creatinine ratio (nmol:mmol) | Mean | 4.7 | 4.6 | 16.0 | 33.6 | 10.3 | 19.1 |
| | SD | 2.7 | 3.7 | 8.1 | 35.6 | 8.3 | 28.9 |
| | *P* | 0.71 | | 0.38 | | 0.84 | |
| MN:creatinine ratio (nmol:mmol) | Mean | 24.6 | 28.9 | 56.2 | 94.7 | 40.4 | 61.8 |
| | SD | 18.6 | 15.7 | 26.4 | 53.5 | 27.5 | 51.2 |
| | *P* | 0.34 | | 0.042 | | 0.15 | |
| NMN:creatinine ratio (nmol:mmol) | Mean | 57.8 | 65.6 | 150.4 | 277.7 | 104.1 | 171.7 |
| | SD | 33.1 | 44.2 | 72.3 | 273.7 | 72.5 | 220.4 |
| | *P* | 0.94 | | 0.41 | | 0.62 | |

M = male, F = female, EPI = epinephrine, NE = norepinephrine, MN = metanephrine, NMN = normetanephrine, SD = standard deviation

**Table 4.** Associations between case outcome and catecholamine and metanephrine:creatinine ratios in Group 2

| **Association with case outcome** | | **Group 2** | |
|---|---|---|---|
| | **Outcome** | **S** | **NS** |
| | *N* | 20 | 5 |
| EPI:creatinine ratio (nmol:mmol) | Mean | 7.6 | 8.4 |
| | SD | 7.1 | 6.2 |
| | *P* | 0.48 | |
| NE:creatinine ratio (nmol:mmol) | Mean | 21.4 | 40.2 |
| | SD | 17.9 | 50.9 |
| | *P* | 0.40 | |
| MN:creatinine ratio (nmol:mmol) | Mean | 73.2 | 88.1 |
| | SD | 40.6 | 69.1 |
| | *P* | 0.92 | |
| NMN:creatinine ratio (nmol:mmol) | Mean | 185.9 | 339.4 |
| | SD | 135.3 | 175.4 |
| | *P* | 0.30 | |

S = survivor; NS = nonsurvivor; EPI = epinephrine; NE = norepinephrine; MN = metanephrine; NMN = normetanephrine; SD = standard deviation

APPENDIX D: RAW DATA

**Table 5.** Catecholamine and metanephrine concentrations in a pooled urine sample, measured immediately after collection (Day 1) and after freezing at -70°C for up to 78 days.

| Day | EPI (nmol) | NE (nmol) | MN (nmol) | NMN (nmol) |
|---|---|---|---|---|
| 1 | 9.7 | 23.3 | 250 | 687 |
| 1 | 9.4 | 22.1 | 258 | 660 |
| 1 | 10.2 | 23.9 | 306 | 692 |
| 1 | 9.6 | 22.1 | 298 | 742 |
| 1 | 10.4 | 22.3 | 297 | 556 |
| 1 | 11.0 | 21.7 | 282 | 700 |
| 1 | 10.0 | 24.1 | 288 | 703 |
| 1 | 12.2 | 22.5 | 296 | 711 |
| 3 | 13 | 34 | 307 | 731 |
| 3 | 14 | 33 | 313 | 761 |
| 5 | 11 | 28 | 265 | 702 |
| 5 | 11 | 28 | 271 | 746 |
| 8 | 21 | 27 | 285 | 813 |
| 8 | 28 | 34 | 263 | 785 |
| 11 | 11 | 25 | 277 | 761 |
| 11 | 11 | 25 | 255 | 744 |
| 12 | 11 | 27 | 325 | 859 |
| 12 | 12 | 28 | 304 | 844 |
| 15 | 12 | 27 | 281 | 781 |
| 15 | 13 | 26 | 299 | 797 |
| 25 | 11 | 29 | 189 | 587 |
| 25 | 13 | 31 | 203 | 617 |
| 50 | 13 | 27 | No recovery | No recovery |
| 50 | 14 | 27 | No recovery | No recovery |
| 78 | 13 | 25 | 300 | 605 |
| 78 | 13 | 25 | 283 | 629 |

EPI = epinephrine; NE = norepinephrine; MN = metanephrine; NMN = normetanephrine

**Table 6.** Catecholamine and metanephrine:creatinine ratios in eight dogs, measured at multiple time points in the course of a day.

| Study Dog | Time (hours) | Epi:Creat (nmol:mmol) | NE:creat (nmol:mmol) | MN:creat (nmol:mmol) | NMN:creat (nmol:mmol) |
|---|---|---|---|---|---|
| 15H | 0 | 0.89 | 2.3 | 28.9 | 53.16 |
| | 3 | 2.5 | 5.7 | 41.6 | 80.7 |
| | 6 | 1.3 | 3.7 | 34 | 71.09 |
| | 9 | 1.9 | 4.76 | 39.06 | 74.39 |
| 22H | 0 | 1.1 | 1.9 | 24.7 | 54.94 |
| | 3 | 6.8 | 6 | 39.5 | 61.4 |
| | 8 | 5.05 | 4.94 | 32.7 | 59.84 |
| | 11 | 1.3 | 2.3 | 26.9 | 57.89 |
| 26H | 0 | 2 | 2.4 | 8.4 | 36.5 |
| | 3 | 2.4 | 3.4 | 11.2 | 32.3 |
| | 6 | 1.7 | 2.9 | 10.1 | 36 |
| | 9 | 0.68 | 2.4 | 8.24 | 33.6 |
| 28H | 0 | 4.9 | 12 | 52.5 | 70.1 |
| | 3 | 3.6 | 9.6 | 53 | 79.7 |
| | 6 | 1.6 | 7 | 42.5 | 74 |
| | 9 | 2.7 | 5.3 | 46.8 | 73.68 |
| 31H | 0 | 2.5 | 3.1 | 23.6 | 46.8 |
| | 3 | 7.8 | 9.2 | 29.6 | 54.7 |
| | 6 | 3.5 | 3.9 | 31 | 57.08 |
| | 9 | 1.7 | 2.2 | 37 | 64.15 |
| 37S | 0 | 1.8 | 29.3 | 56.6 | 269 |
| | 4 | 1.7 | 22.8 | 43.4 | 259.9 |
| | 8 | 2 | 18.7 | 44.2 | 196.7 |
| | 12 | 2.2 | 18.7 | 57.1 | 210.4 |
| 38S | 0 | 3.2 | 6.5 | 67.7 | 123 |
| | 4 | 3.8 | 6.8 | 69.3 | 116 |
| | 7.5 | 4.4 | 7.9 | 70.1 | 118 |
| | 11.5 | 4.1 | 7.1 | 67.5 | 122 |
| 5S | 0 | 4.8 | 10 | 44.6 | 113 |
| | 4 | 4.5 | 7.4 | 42.1 | 93.6 |
| | 8 | 5.1 | 9 | 48 | 100 |
| | 11 | 3.2 | 7 | 33.3 | 92.2 |

EPI = epinephrine; NE = norepinephrine; MN = metanephrine; NMN = normetanephrine; Creat = creatinine

**Table 7.** Descriptive data for all study dogs

| Study Dog | Group* | Breed | Age (years) | Gender[%] | Body Weight (kg) |
|---|---|---|---|---|---|
| 1H | 1 | Labrador retriever | 7 | FS | 27.7 |
| 2H | 1 | Mix | 9 | MC | 37.5 |
| 3H | 1 | Black and tan hound | 7 | FS | 25 |
| 4H | 1 | Mix | 6 | MC | 16 |
| 5H | 1 | Mix | 9 | FS | 28.5 |
| 6H | 1 | Mix | 8 | FS | 25.7 |
| 7H | 1 | Mix | 8 | FS | 30.3 |
| 8H | 1 | Australian cattle dog | 15 | MC | 29.7 |
| 9H | 1 | Mix | 6 | FS | 17.1 |
| 12H | 1 | Labrador retriever | 12 | MC | 33.8 |
| 14H | 1 | Mix | 7 | MC | 15.9 |
| 17H | 1 | Border collie | 8 | FS | 17.5 |
| 18H | 1 | Labrador retriever | 11 | MC | 40.9 |
| 20H | 1 | Norwish terrier | 12 | FS | 5 |
| 21H | 1 | Mix | 5 | FS | 11.6 |
| 22H | 1 | Mix | 10 | FS | 26 |
| 23H | 1 | Mix | 10 | MC | 12.3 |
| 24H | 1 | Australian shepherd | 6 | FS | 31.8 |
| 25H | 1 | Mix | 8 | MC | 28.2 |
| 26H | 1 | Labrador retriever | 9 | MC | 27.3 |
| 27H | 1 | Golden retriever | 6 | FS | 37.3 |
| 28H | 1 | Mix | 9 | FS | 21.8 |
| 29H | 1 | Mix | 9 | MC | 35.9 |
| 31H | 1 | Mix | 9 | MC | 37.2 |
| 32H | 1 | Golden retriever | 9 | MC | 36.8 |
| 5S | 2 | Labrador retriever | 6 | FS | 30.2 |
| 7S | 2 | Scottish terrier | 12 | MC | 12.2 |
| 11S | 2 | Mix | 8 | FS | 7.2 |
| 12S | 2 | Lab | 10 | MC | 39 |
| 13S | 2 | Shih tzu | 13 | FS | 4 |
| 14S | 2 | Boxer | 10 | MI | 29.8 |
| 15S | 2 | Border collie | 10 | FS | 18.4 |
| 16S | 2 | Pomeranian | 7 | FS | 4.4 |
| 18S | 2 | Shih tzu | 9 | MC | 8.6 |
| 19S | 2 | Dachshund | 10 | FS | 5.3 |
| 22S | 2 | Bernese mountain dog | 6 | FS | 27.3 |
| 23S | 2 | Shetland sheepdog | 8 | FS | 16.8 |
| 24S | 2 | Labrador retriever | 12 | MC | 33.9 |
| 26S | 2 | Shetland sheepdog | 8 | MC | 16.4 |
| 27S | 2 | Mix | 6 | FS | 19.5 |

| | | | | | |
|---|---|---|---|---|---|
| 29S | 2 | Beagle | 5 | FS | 13.5 |
| 30S | 2 | Mix | 10 | MC | 26.4 |
| 32S | 2 | Cocker spaniel | 6 | FS | 16.1 |
| 33S | 2 | Beagle | 9 | MC | 8.7 |
| 34S | 2 | Mix | 13 | MC | 31.2 |
| 35S | 2 | Bichon frise | 12 | MC | 9.5 |
| 36S | 2 | Dachshund | 5 | MC | 7.2 |
| 37S | 2 | Shih tzu | 11 | FS | 4.9 |
| 39S | 2 | Mix | 7 | MC | 33.2 |
| 41S | 2 | Weimaraner | 6 | FS | 32 |

*1 = healthy dogs; 2 = critically ill dogs
% FS = female, spayed; MC = male, castrated; MI = male, intact

**Table 8.** Systolic blood pressure and average adrenal gland size for all study dogs

| Study Dog | Group* | systolic blood pressure (mmHg) | average adrenal gland size (mm) |
|---|---|---|---|
| 1H | 1 | 110 | 5.1 |
| 2H | 1 | 150 | 5.8 |
| 3H | 1 | 110 | 4.4 |
| 4H | 1 | 130 | 5.55 |
| 5H | 1 | 115 | 6.3 |
| 6H | 1 | 115 | 6.8 |
| 7H | 1 | 120 | 4.95 |
| 8H | 1 | 125 | 6.25 |
| 9H | 1 | 170 | 4.8 |
| 12H | 1 | 125 | 6.3 |
| 14H | 1 | 145 | 5.25 |
| 17H | 1 | 110 | 4.75 |
| 18H | 1 | 125 | 7.15 |
| 20H | 1 | 180 | 4.8 |
| 21H | 1 | 110 | 4.3 |
| 22H | 1 | 130 | 7.35 |
| 23H | 1 | 195 | 5.1 |
| 24H | 1 | 120 | 5.85 |
| 25H | 1 | 145 | 5.15 |
| 26H | 1 | 125 | 5.3 |
| 27H | 1 | 150 | 5.9 |
| 28H | 1 | 120 | 5.5 |
| 29H | 1 | 150 | 5.75 |
| 31H | 1 | 115 | 7 |
| 32H | 1 | 160 | 5.6 |
| 5S | 2 | 150 | 4.5 |
| 7S | 2 | 120 | 5.75 |
| 11S | 2 | 105 | 5.1 |
| 12S | 2 | 130 | NA |
| 13S | 2 | 110 | 4.2 |
| 14S | 2 | 90 | 6.35 |
| 15S | 2 | 173 | 3.95 |
| 16S | 2 | 242 | 5.05 |
| 18S | 2 | 230 | 4.65 |
| 19S | 2 | 120 | 4.4 |
| 22S | 2 | 150 | 5 |
| 23S | 2 | 130 | 6.55 |
| 24S | 2 | 110 | 7.4 |
| 26S | 2 | 110 | 3.5 |
| 27S | 2 | 135 | 6.35 |

| | | | |
|---|---|---|---|
| 29S | 2 | 90 | 4.3 |
| 30S | 2 | 120 | 6 |
| 32S | 2 | 150 | 3.8 |
| 33S | 2 | 110 | 4.55 |
| 34S | 2 | 75 | NA |
| 35S | 2 | 170 | 4.4 |
| 36S | 2 | NA | 5.7 |
| 37S | 2 | 150 | 5.7 |
| 39S | 2 | 120 | 5.45 |
| 41S | 2 | 135 | 6.75 |

NA = not available

*1 = healthy dogs; 2 = critically ill dogs

**Table 9.** Primary disease process and outcome of critically ill dogs

| Study Dog | Disease process | Outcome* |
|---|---|---|
| 5S | pancreatic | S |
| 7S | neoplasia | S |
| 11S | pancreatic | S |
| 12S | hepatobiliary | S |
| 13S | hepatobiliary | S |
| 14S | neoplasia | NS |
| 15S | neurologic | S |
| 16S | immune-mediated | NS |
| 18S | immune-mediated | S |
| 19S | gastrointestinal | S |
| 22S | immune-mediated | S |
| 23S | hepatobiliary | S |
| 24S | respiratory | S |
| 26S | pancreatic | NS |
| 27S | hepatobiliary | NS |
| 29S | immune-mediated | S |
| 30S | neurologic | S |
| 32S | immune-mediated | S |
| 33S | respiratory | S |
| 34S | neoplasia | NS |
| 35S | gastrointestinal | S |
| 36S | immune-mediated | S |
| 37S | immune-mediated | S |
| 39S | neurologic | S |
| 41S | gastrointestinal | S |

* S = survived until discharge; NS = nonsurvivor

**Table 10.** Catecholamine and metanephrine:creatinine ratios in individual dogs

| Study Dog | Group* | EPI:Creat (nmol:mmol) | NE:creat (nmol:mmol) | MN:creat (nmol:mmol) | NMN:creat (nmol:mmol) |
|---|---|---|---|---|---|
| 1H | 1 | 1.2 | 1.6 | 20.4 | 33.9 |
| 2H | 1 | 2.12 | 5.38 | 24.66 | 52.82 |
| 3H | 1 | 0.6 | 0.6 | 17.3 | 46.1 |
| 4H | 1 | 2.7 | 3.1 | 35.72 | 60.18 |
| 5H | 1 | 5.9 | 7.06 | 30.8 | 64.04 |
| 6H | 1 | 1.8 | 3 | 26.9 | 51.79 |
| 7H | 1 | 1 | 1.5 | 15.1 | 33.2 |
| 8H | 1 | 11 | 11 | 78 | 148 |
| 9H | 1 | 1.2 | 2.6 | 21.1 | 42.41 |
| 12H | 1 | 1.6 | 7.86 | 17.1 | 81.4 |
| 14H | 1 | 0.52 | 0.97 | 15.4 | 34.28 |
| 17H | 1 | 6.34 | 6.64 | 43.27 | 77.05 |
| 18H | 1 | 4.2 | 5 | 25.5 | 58.6 |
| 20H | 1 | 7.8 | 11.6 | 63.2 | 130 |
| 21H | 1 | 2.4 | 4.5 | 34.2 | 183 |
| 22H | 1 | 1.1 | 1.9 | 24.7 | 54.94 |
| 23H | 1 | 3.83 | 7.45 | 25.6 | 73.35 |
| 24H | 1 | 2.7 | 3.46 | 15.4 | 37.71 |
| 25H | 1 | 2 | 2.8 | 7.24 | 22.5 |
| 26H | 1 | 2 | 2.4 | 8.4 | 36.5 |
| 27H | 1 | 3.3 | 3.91 | 10.6 | 28.9 |
| 28H | 1 | 4.9 | 12 | 52.5 | 70.1 |
| 29H | 1 | 1.3 | 2.6 | 20.1 | 35.26 |
| 31H | 1 | 2.5 | 3.1 | 23.7 | 46.8 |
| 32H | 1 | 1.8 | 4.4 | 14.2 | 44.5 |
| 5S | 2 | 4.9 | 10 | 42.3 | 103 |
| 7S | 2 | 6.3 | 26.6 | 85.6 | 227 |
| 11S | 2 | 6.7 | 9.4 | 68.2 | 95.3 |
| 12S | 2 | 5.8 | 13 | 45.4 | 110 |
| 13S | 2 | 6.1 | 29.5 | 68.9 | 240 |
| 14S | 2 | 1.1 | 12.3 | 40.6 | 190.4 |
| 15S | 2 | 14 | 15.6 | 61.65 | 102.2 |
| 16S | 2 | 18 | 131 | 208 | 1040 |
| 18S | 2 | 5.5 | 7.3 | 48.9 | 72.6 |
| 19S | 2 | 9.5 | 8.9 | 125 | 145 |
| 22S | 2 | 16 | 59.6 | 128 | 465.9 |
| 23S | 2 | 5.6 | 5.9 | 56.9 | 67.1 |
| 24S | 2 | 2.1 | 26.5 | 37.9 | 290.7 |
| 26S | 2 | 7.1 | 17.1 | 51.2 | 158.5 |
| 27S | 2 | 10 | 24.3 | 85.3 | 132 |

| | | | | | |
|---|---|---|---|---|---|
| 29S | 2 | 4.7 | 35.8 | 121 | 428.8 |
| 30S | 2 | 4.69 | 9.94 | 42.7 | 96.83 |
| 32S | 2 | 2.1 | 6.87 | 32.2 | 60.9 |
| 33S | 2 | 8.3 | 19.4 | 85.4 | 141.3 |
| 34S | 2 | 6.03 | 16.3 | 55.3 | 176 |
| 35S | 2 | 8 | 29.6 | 116 | 213.9 |
| 36S | 2 | 5 | 7 | 44.2 | 58.1 |
| 37S | 2 | 1.8 | 29.3 | 56.6 | 269 |
| 39S | 2 | 1.3 | 6.9 | 20.6 | 69.8 |
| 41S | 2 | 33.2 | 70.79 | 176.7 | 460.7 |

EPI = epinephrine; NE = norepinephrine, MN = metanephrine, NMN = normetanephrine, Creat = creatinine
*1 = healthy dogs; 2 = critically ill dogs