SYMMETRIC DIMETHYLARGININE: A NOVEL RENAL BIOMARKER

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A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Clinical Sciences
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2016

Approved by:

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Abstract

Chronic kidney disease (CKD) is a potentially life-threatening disease that reportedly affects 10% of dogs and 30% of cats over the age of 15. There is no cure available for CKD, but medical management is available for patients with this disease. Research has focused on earlier detection of CKD with the goal of instituting medical management and monitoring as early in the disease course as possible. Symmetric dimethylarginine (SDMA) has recently emerged as a novel renal excretory biomarker that may aid in early detection of CKD in cats and dogs. SDMA is non-protein bound and is freely filtered by the glomerulus, is not secreted or reabsorbed, and has greater than 90% excretion by the kidneys, making it a potential target for measurement of glomerular filtration rate (GFR). Previous studies have demonstrated a close parallel between SDMA and serum creatinine (sCr), which is the currently favored serum biomarker for assessment of GFR. Research has also demonstrated a correlation between SDMA and GFR. Serum concentrations of SDMA increase above normal when GFR is decreased by 25-40%; much earlier than the 75% decrease in GFR typically required for sCr to increase above its reference interval. The studies reported here demonstrate a potential use for the SDMA:sCr ratio as a predictor of volume responsive azotemia. Furthermore, longitudinal assessment of older dogs and cats for early detection of CKD showed that SDMA was a more sensitive indicator of CKD than sCr. The evaluation of SDMA reported in this thesis presents a novel perspective on SDMA and its use clinically.

Table of Contents

List of Tables and Figures

Chapter 1 - Symmetric dimethylarginine – Literature Review and Relationship to Chronic Kidney Disease

Introduction

Symmetric dimethylarginine (SDMA) was first identified in 1970 by Kakimoto and Akazawa as a urine metabolite and was suspected to have a functional role in protein synthesis.[1] Protein synthesis occurs in the nucleus of cells via several steps. The first is transcription, in which a messenger ribonucleic acid (mRNA) chain is generated from the deoxyribonucleic acid chain that makes up the genetic blueprint of the organism. Once the mRNA chain is formed, it is translated in the cytoplasm into an amino acid chain. This amino acid chain is the foundation of the protein. After translation, the amino acids undergo post-translational modification and residues (individual amino acids) are altered by enzymes that cause (among other effects) methylation, glycosylation, and phosphorylation. This post-translational modification influences protein folding and, ultimately, the structure of the protein itself.[2]

It is now known that SDMA and a similar protein, asymmetric dimethylarginine (ADMA) are derived from posttranslational modification (methylation) of arginine residues within almost every cell. After proteolysis, or protein breakdown, these protein residues are released into the circulation. (Figure 1.1) The biological importance of SDMA and ADMA has not been fully resolved, but ADMA has been linked to inhibition of nitric oxide synthase and SDMA has been shown to interfere with amino acid uptake by endothelial cells, but only in vitro and at supra-physiologic concentrations.[3,4] However, the potential use of serum SDMA as a marker for evaluation of kidney disease, cardiovascular health, atherosclerosis, rheumatoid arthritis, and other diseases, has been extensively studied. [4,5,6]

SDMA is eliminated from the body primarily via renal excretion (>90% via glomerular filtration without tubular reabsorption or secretion), but some small percentage of its elimination may be associated with non-renal enzymatic cleavage and subsequent degradation. SDMA does not bind to protein, unlike ADMA, which is partially bound to plasma proteins. Serum concentrations of SDMA are increased in human patients with chronic kidney disease (CKD) and it has been shown that serum SDMA concentrations are inversely correlated with glomerular

filtration rate (GFR).[7] Correlations between ADMA and GFR have been shown to be much weaker, likely due to the protein-bound fraction of ADMA, which hinders glomerular filtration.[7]

Asymmetric Dimethylarginine

The reaction that converts a post-translational methylated protein to SDMA is catalyzed by protein-arginine methyltransferase (PRMT) and additionally produces a separate naturally occurring amino acid derivative known as ADMA. This occurs in every nucleated cell in the body as part of normal post-translational protein modification and subsequent proteolysis. The location of the methylated nitrogen on the arginine residues determines whether the amino acid derivative bi-product will be symmetric or asymmetric after proteolysis occurs, producing either SDMA or ADMA. ADMA has many functions in the body, but is most well known as a potent competitive inhibitor of nitric oxide synthases (NOS). Importantly, ADMA is partially protein-bound and therefore is less efficiently eliminated via glomerular filtration.[3,8] Nitric oxide (NO) is continually produced in many locations throughout the body and functions mainly as a vasodilator, with direct action on vascular endothelial cells. NO is also an inhibitor of platelet adhesion and aggregation. NO is produced from several precursors, one of which is L-arginine, which is converted to NO by NOS.[9] It is a key regulatory molecule for vascular tone, blood pressure, and blood flow. Because ADMA inhibits NOS, it has been hypothesized that increased circulating serum concentrations of ADMA may contribute to systemic hypertension in patients with CKD associated with accumulation of ADMA.[10] NO has functions (mostly involving vasodilation) in the brain, peripheral nervous system, and virtually all other organ systems in the body, including the the immune system, and therefore the accumulation of ADMA in patients with CKD potentially plays a role in the pathogenesis of CKD.

Symmetric Dimethylarginine

The ratio of ADMA to SDMA produced depends on the residues present on the original modified protein.[1] Distinguishing SDMA from its structural isomer, ADMA, has important implications in overall assessment of these residues because SDMA appears to be less biologically active compared with ADMA. The functional role of SDMA and whether SDMA

also inhibits NOS is controversial.[5,8] It has been proposed that the effects of SDMA on NO production are due to inhibition of L-arginine uptake rather than direct inhibition of NOS.[6]

SDMA as a Renal Biomarker

SDMA and ADMA are both protein residues that have similar production pathways, but differ in importance with regard to CKD. SDMA is more promising renal function biomarker because it is more strongly correlated to GFR and appears to have limited biologic activity elsewhere in the body. Serum SDMA concentrations are known to be increased in CKD patients and SDMA has potentially been linked to chronic inflammation in CKD patients, but the biologic significance remains controversial. *In vitro* SDMA stimulated production of reactive oxygen species in monocytes.[11] SDMA concentrations correlate with interleukin-6 (IL-6), tumor necrosis factor - α (TNF- α), and albumin in human patients with CKD,[7] whereas ADMA does not appear to be associated with these inflammatory molecules.[12] This means that SDMA is not only a molecule of importance for measurement and evaluation of renal excretory function in CKD, but perhaps increased levels of SDMA can be linked to inflammation and progression of CKD. Serum concentrations of SDMA may be increased in other pathological or physiologic events and its exclusive elimination by the kidneys has never been unequivocally proven. However, studies have shown that serum SDMA concentrations closely parallel GFR and associations between SDMA and other pathophysiologic events unrelated to kidney disease are lacking in the human literature. In a meta-analysis performed by Kielstein and colleagues in 2006, SDMA was evaluated as a renal excretory marker using all studies published from 1970 to 2006. In 18 studies involving 2136 patients, systemic SDMA concentrations were highly correlated with inulin clearance ($R = 0.85$), as well as with other methods of GFR measurement such as Cockroft-Gault GFR measurements and creatinine clearance testing ($R = 0.77$). SDMA also correlated well with serum creatinine ($R = 0.75$). Conversely, ADMA showed minimal correlation to creatinine and minimal correlation to inulin clearance tests. Measurement of GFR is not always practical in the clinical setting and surrogate markers of GFR like sCr are frequently evaluated instead. Serum SDMA concentrations tend to correlate better with GFR than do sCr concentrations in people perhaps because sCr is influenced by the patient's muscle

mass and in some cases diet.[7] Hence, there is increasing interest in SDMA as a surrogate GFR marker in people.

Chronic Kidney Disease in Veterinary Medicine

CKD is a common problem in dogs and cats that increases in prevalence with age. Over 10% of dogs and greater than 30% of cats over the age of 15 years were diagnosed with CKD in one report.[13] CKD is defined as a structural and/or functional abnormality of one or both kidneys that has been continuously present for three months or longer (i.e. long enough for compensatory hypertrophy of the remaining nephrons to occur). Loss of renal function in CKD is a reflection of the ongoing loss of nephrons, which represent the functional unit of the kidney. Overall renal function is the sum of the function of each individual nephron, which is the functional unit of the kidney. In 1960, Bricker and colleagues developed the intact nephron hypothesis, which states "as the number of functioning nephrons decreases, each remaining nephron must perform a greater fraction of total renal excretion".[14] As nephrons are lost with CKD progression, remaining nephrons undergo compensatory hypertrophy, resulting in increased intraglomerular pressure and hyperfiltration, which can lead to additional nephron damage and loss. This process is called spontaneous progression of CKD. Although the exact mechanisms for continuing nephron death are unknown, it is hypothesized that inflammation and potentially, adaptive changes imposed by the need for increased individual nephron function contribute to ongoing nephron loss.[14,15] This hypothesis of progression of CKD includes six sequential steps.[14,15,16] In the first step, glomerular injury produces local, intrarenal hypertension, which increases the GFR that must be maintained by that individual nephron (single nephron GFR, [SNGFR]). Local hypertension then leads to protein leakage and proteinuria, as well as induction of inflammatory cytokines and subsequent accumulation of mononuclear cells. In step three, neutrophils, lymphocytes, and macrophages infiltrate the area, causing renal inflammation. Steps 4-6 are associated with derangements in the renal tubular epithelial cells and their basement membranes, fibrosis, and eventually replacement of the nephron with scar tissue.[14,15,16]

Absent a known sustained acute kidney injury event, the initial etiology of CKD is usually unknown. Attempts have been made to identify a genetic component that would lead to

spontaneous progressive nephron loss in dogs and cats, but no definitive evidence exists for this theory.[16] Other proposals have been made for causes for CKD, including vaccines, periodontal disease, unknown chronic toxicant ingestion, and feline immunodeficiency virus (FIV), but conclusive evidence to support these theories is not currently available.[16] Further research is needed to understand the onset and initial inciting cause of chronic kidney disease in cats and dogs.

Veterinary guidelines for the classification and staging of CKD have been developed by the International Renal Interest Society (IRIS).[17] (Table 1.1 and Table 1.2)

Beyond mid-Stage 2, CKD is typically irreversible and potentially progressive. Progression can be slowed with renoprotective therapy, which may include dietary therapy, maintenance of hydration, and careful monitoring and treatment (if needed) of proteinuria and hypertension. Treatment for CKD is described in detail elsewhere, but early detection and treatment may improve the outcome associated with renoprotective therapies.[16,18]

SDMA in Veterinary Medicine

Chronic Kidney Disease and the Need for Early Detection

Similar to previously discussed studies in people, SDMA has been investigated in veterinary medicine. If SDMA proves to be a sensitive and specific biomarker for early canine and feline CKD, improved early diagnosis may result in improved therapeutic outcomes (Figure 1.2).

Assay validation for SDMA

Prior to clinical evaluation of SDMA in dogs and cats, assays for detection of this molecule were validated by IDEXX Laboratories and described by Hall et al in 2014.[19,20,21] The analyte is measured using liquid chromatography/mass spectrometry (LC-MS) using an API 4000 mass spectrometer coupled with the Shimadzu Nexera high performance liquid chromatography (HP-LC) system. The LC-MS standard curve using the protocol prepared for SDMA had a correlation coefficient of >0.999.[19,20] The LC-MS assay was also validated using quantifications of a standard curve, lowest concentration limits, signal-to-noise ratio and minimum acceptable values, sample recovery, dilutional integrity, intra-assay precision, and operator/machine variability using different operators and instruments. These were performed under standard LC-

MS protocol. Furthermore, the effect of interfering substances was also investigated. Additives to the solutions were used to mimic hemolysis, lipemia, and bilirubin. SDMA levels in these solutions, as well as interference with additional similar molecules (monomethylarginine (MMA) and ADMA) was anazlyed. No interference by other molecules was detects and no effect was incurred from the additives.[20] Stability of SDMA in serum or plasma has also been evaluated.[22,23] In a study by Yerramilli in 2013, serum samples were evaluated from tubes containing ethylenediaminetetraacetic acid (EDTA), lithium heparin, and sodium citrate as well as whole blood with EDTA. Three different temperatures (0° C, 4° C, and room temperature) and 3 different concentrations of SDMA were studied to include the effect of freeze, thaw, and sample volume on the SDMA measurements obtained by the previously validated LC-MS assay. The results suggested that SDMA is stable in whole blood, serum and plasma at 4° C and room temperature up to seven days. There are no noticeable differences between collection tubes, serum and plasma. Canine and feline serum samples performed equally well in this assay.[20] To facilitate use in the clinical setting, SDMA concentrations are now measured by automated, high through-put assays developed by IDEXX laboratories that correlates well with the LC-MS methodology. Further assay validation and development regarding a bench-top test or including SDMA on the in-house laboratory machines is a possibility for the future.

Comparison of SDMA to Standard Measurements for Determining GFR in Dogs and Cats

Nabity *et al* (2015) compared iohexol clearance, serum creatinine, and SDMA measurements for evaluation of GFR using previously described methodology.[20,23,24] Dogs (n=8) with X-linked hereditary nephropathy (XLHN) were studied and 4 unaffected female littermates were used as controls. XLHN is a genetic defect that causes rapidly progressive kidney disease. Males with this mutation succumb to end-stage renal disease before 18 months of age.[35] This makes XLHN a model for study of CKD and female littermates an ideal control group. Blood and urine was collected on a weekly basis starting at 7 weeks of age. GFR (via iohexol plasma clearance) was determined monthly. Three of the 8 XLHN dogs were euthanized before the study endpoint, which was determined prior to the start of the study as the point at which the XLHN dogs would reach a serum creatinine (sCr) of greater than 5 mg/dL. This is

expected to occur in XLHN dogs prior to 18 months of age. In affected dogs, both sCr and SDMA had a strong correlation with GFR (r= -0.98 and -0.95, respectively). However, in the unaffected dogs, SDMA did not correlate with GFR, sCr, age, or weight. The reason for this is unknown, but the authors hypothesized that there may be an increase in SDMA production in juveniles secondary to growth. In affected dogs, SDMA increased earlier than sCr. SDMA did intermittently increase in unaffected dogs, but had a subsequently normal value and increase in SDMA was only deemed important if it was persistently increased. It can therefore be inferred that a single, one-time, elevation in SDMA in a dog that has no clinical signs or other laboratory abnormalities consistent with CKD should be interpreted with caution. In this case, it would be prudent for the clinician to simply recheck blood work (including SDMA) in a few months. There were no cases in the Nabity study of elevated creatinine without concurrent elevation in SDMA. The authors concluded that SDMA was a useful test for identifying and monitoring renal function in dogs.[20,23,24] This study also corroborated previous SDMA LC-MS validation and confirmed the stability of SDMA in canine blood. Results of the SDMA assay validation showed an average accuracy of 101% with 95-98% sample recovery, 95-107% dilutional integrity, and nearly identical inter-operator and instrument results. Furthermore, blood samples exposed to 3 freeze-thaw cycles were found to be equivalent.[20,21,22,23]

In 2014, Braff and colleagues showed that increased serum SDMA concentrations in cats correlated with decreased GFR.[25] Ten client-owned cats underwent plasma iohexal clearance testing for GFR determination and also had SDMA measured by LCMS assay. SDMA correlated well with GFR in an inverse linear relationship (R= -0.82). Creatinine was also measured and performed equally well (R= -0.81). Although a strong correlation was shown between serum SDMA concentrations and plasma clearance of iohexol, the sample size was small.[25]

Hall *et al* showed in 2014 that SDMA is potentially more sensitive than serum creatinine for early detection of CKD in cats.[19] Twenty-one cats with CKD (defined as persistent increases sCr > 2.1mg/dL for greater than three months (n=15), or a decline in GFR that was greater than 30% below the median GFR (n=4), and 2 nonazotemic cats with calcium oxalate stones) were compared to 21 healthy cats using retrospective data over a period of years. In this study, azotemia was defined as a sCr value greater than 2.1 mg/dL (based on the laboratory's reference interval) and an abnormal SDMA was defined as > 14 μg/mL (based on previous Idexx studies).

SDMA and sCr correlated with GFR (r = -0.79 and -0.77, respectively). Serum SDMA concentrations increased prior to increased sCr concentrations in 17/21 cats. SDMA showed a higher sensitivity (100%) than sCr (17%) but lower specificity (91%, versus 100% for creatinine) for detection of CKD. The proposed cause for the lower specificity of SDMA was due to the definition of reduced GFR (> 30% decrease from median). There were two cats with SDMA >14 ug/dL in this study that did not have a GFR value that was low enough to meet the predetermined cutoff value for reduced kidney function. However, both of these cats had a 25% decrease in GFR compared to the reference interval and had they been included in the CKD group, SDMA would have had a 100% specificity for detection of CKD. The sensitivity of measuring sCr concentrations for early detection of CKD can be increased if a lower threshold for azotemia was employed (e.g., 1.6 mg/dl vs. 2.1 mg/dl). In this study, if a 1.6 mg/dl sCr limit had been used, the sensitivity for creatinine for detection of CKD would have been 50% instead of 17%, which is still lower than the sensitivity of SDMA for detection of CKD.

A similar study was performed in dogs.[26] Over a period of 3 years, older dogs that developed CKD (based on plasma iohexol clearance data) had increases in serum SDMA earlier compared with increased sCr concentrations.[26] In a separate but related study, concentrations of SDMA were evaluated in canine and feline patients that had increases in sCr over time, but within reference intervals.[27] All of these patients had not yet developed azotemia (based on the laboratory's reference interval), but individual's reference intervals were calculated based on the average sCr for that patient over several years (patients were used that had four or more sCr samples available). Thirty-nine canine patients and 48 feline patients were selected for analysis whose sCr had never exceeded the individual limit (Group 1). Group one was compared to 38 canine patients and 33 feline patients whose sCr did exceed the individual reference interval yet remained within the reference range (Group 2). The canine patients in group 1 that had stable sCr had an elevated SDMA (> 14μg/dL) 17.9% of the time, whereas the patients in group 2 that had increases in sCr over time (but still within the reference interval) had elevated SDMA 63.2% of the time. For the feline patients, 8.3% of the stable sCr group had increased serum SDMA concentration, but 42.4% of the trending increase in sCr group had increased serum SDMA concentrations. The study showed that SDMA was above the reference limits more frequently for canine and feline patients whose sCr may be increasing over time, yet remained within normal reference limits than in patients exhibiting stable creatinine concentrations that were not

increasing above the individual limits.[26,27]

Hall and colleagues also published a study that compared serum SDMA and sCr in healthy geriatric cats fed a diet with reduced protein and added fish oil, L-carnitine, and medium-chain triglycerides (MCTs).[28] The purpose was to evaluate whether this diet affected serum biomarkers for CKD, such as SDMA and sCr. Thirty-two cats were divided into three groups and fed either the control diet, a diet enhanced with added fish oil and L-carnitine, or a diet enhanced with added fish oil and L-carnitine and higher MCTs, plus corn oil and lower animal fat for six months. The diets were similar in moisture concentration, and protein and fat content. No changes in renal biomarkers were observed, but the study concluded that assessment of serum SDMA concentration was a better marker of GFR than was sCr concentration in geriatric cats with a lower lean body mass.[28] This was evidenced by the fact that compared with cats less than 12 years of age, cats greater than 15 years old had lower lean body mass, lower GFR, and lower sCr, but higher SDMA concentrations, which were also more closely negatively correlated with GFR (R = - 0.72 versus -0.5 for creatinine). The decreases in lean body mass that potentially impacted sCr concentrations did not appear to impact serum SDMA concentrations.

One of the major criticisms of using sCr to evaluate GFR is the potentially confounding relationship between sCr and lean body mass. As lean body mass declines with age and/or secondarily to chronic disease, serum creatinine concentration and GFR correlation may decline.[16] In 2015, Hall and colleagues evaluated the relationship between lean body mass and serum renal biomarkers in healthy dogs.[29] Forty-one healthy beagles were included and SDMA and creatinine were measured at 1, 3, and 6 months. Lean body mass and age were weakly correlated with sCr (R = 0.38), but no correlation was shown between lean body mass or age and SDMA. The study concluded that lean body mass and age were significant variables for sCr concentrations, but not for serum SDMA concentrations.[29]

CKD is a common, irreversible, and potentially progressive disease in dogs and cats. Early detection is a necessary step for early intervention. Inasmuch as renal replacement therapy is not widely available in veterinary medicine, early detection of CKD is important if we are to slow the rate of progression of CKD and prolong quality of life (Figure 1.2). The ideal biomarker for early CKD detection would be inexpensive to measure, safely and non-invasively obtained from patients, it would be sensitive and specific for CKD, and it would perform better than current biomarkers for both early detection as well as predicting progression. Based on

previous studies in cats and dogs with CKD, evaluation of serum SDMA concentrations has the potential to improve our current diagnostic capabilities. Measurement of serum SDMA concentrations has recently become more widely available, is inexpensive, and its measurement has been extensively validated and found to be accurate. For these reasons, further research in animals with different disease states is warranted, including longitudinal data acquisition in client-owned animals, well as evaluation of serum SDMA in patients with azotemia of unknown cause.

Chapter 2 - The effect of volume responsive dehydration on symmetric dimethylarginine (SDMA) and serum creatinine (sCr) concentrations in dogs and evaluation of the SDMA:sCr ratio

Introduction

Symmetric dimethylarginine (SDMA) was first identified in 1970 by Kakimoto and Akazawa as a urine metabolite.[1] SDMA is one of several methylated arginines that are derived from posttranslational modification of arginine by methylation.[5,8] SDMA is released from cells into the circulation after proteolysis. SDMA is mainly eliminated via glomerular filtration, and therefore serves as a potential marker for kidney function.[8,11] SDMA concentrations have traditionally been measured using LC/MS methodology that has been validated by Padmanabahn and colleagues through IDEXX laboratories.[21,22] The correlation between SDMA and GFR has been established.[19,23,28] SDMA correlated well with GFR and it has been suggested that interpretation of renal excretory function is improved when both SDMA and serum creatinine concentrations (sCr) are assessed along with urine specific gravity (USG).[23] The relationship between SDMA and sCr in cases of volume responsive/pre-renal azotemia however, has not been evaluated.

When 67% of nephrons are lost, the urine that is produced by the kidneys can no longer be maximally concentrated (hypersthenuria).[16,30,31] When nephron loss exceeds 75%, azotemia develops.[30,31] Azotemia is classically divided into three categories: pre-renal, renal, and post-renal azotemia. Pre-renal azotemia is often associated with decreased renal perfusion and renal hemodynamic compromise.[2,16] A functional decline in glomerular filtration results from deficient renal blood flow, decreased perfusion pressure, or excessive renal vasoconstriction.[2,16] The kidneys attempt to conserve water by producing hypersthenuric urine and therefore the distinction between pre-renal and renal azotemia is often aided by evaluation of USG. Renal azotemia implies intrinsic renal damage/disease and is the primary type of azotemia observed in patients with AKI and CKD. Pre-renal azotemia may contribute to the severity of the azotemia in CKD and AKI and there is often some degree of overlap between pre-renal and renal azotemia.[2,16]

Differentiation of pre-renal azotemia from renal azotemia is difficult if USG is unknown (pre-treatment urine sample was not obtained) or if pre-renal azotemia occurs concurrently with a non-renal disorder associated with a urine-concentrating deficit (e.g., glucocorticoid or diuretic treatment, hypoaldosteronism (e.g., Addison's disease), and hypercalcemia). Furthermore, assessment of dehydration in an individual dog or cat can be challenging and typically based on subjective parameters such as skin tent, capillary refill time, and mucus membrane color or tackiness. This makes development of the initial fluid plan imprecise and necessitates frequent evaluation of body weight and signs of fluid overload while the patient is receiving intravenous (IV) fluid therapy. An individual patient's response to volume replacement with fluid therapy (e.g., resolution of the azotemia in cases of volume-responsive azotemia) is one way to evaluate pre-renal azotemia that is superimposed on an inability to concentrate urine. A pre-treatment diagnostic test that could differentiate pre-renal and renal azotemia in cases where a urine sample cannot be obtained, or in cases where urine is minimally concentrated due to presence of another disease could be useful for pre-treatment prognostication.

While SDMA has been shown to correlate with GFR,[19,24] changes in SDMA versus sCr in cases of pre-renal azotemia have not been evaluated clinically. Our objective was to evaluate serum SDMA and sCr in volume responsive azotemia, especially when the pre-renal azotemia is superimposed on an inability to concentrate urine associated with extra-renal disease. Furthermore, a second objective of this study was to evaluate the usefulness of the SDMA:sCr ratio in localization of azotemia. Our hypothesis was that SDMA and sCr will be proportional in dogs and cats with volume-responsive/pre-renal azotemia and that the pre-fluid therapy SDMA:sCr ratio will be similar to post-fluid therapy ratios.

Materials and Methods

Study design

Dogs that were presented to the Veterinary Health Center at Kansas State University with evidence of clinical dehydration were prospectively evaluated. All procedures and study protocols were approved by the Kansas State University Institutional Animal Care and Use Committee and owner consent was obtained for each individual dog enrolled. Prior to initiating fluid therapy, the signalment, presenting clinical complaint, medical history, physical examination findings, and body weight (kg) were recorded and a complete blood count (CBC)[a],

or a packed-call volume and total solids (PCV/TS), serum biochemistry profile[b], and complete urinalysis (UA)[c] or urine specific gravity (USG) were obtained. The patient was then managed at the discretion of the attending clinician with the volume of IV fluids administered based on initial assessment of patient hydration status. A second serum biochemistry profile was assessed 24-48 hours after the start of fluid therapy. Pre- and post-fluid therapy serum samples were banked, frozen, and sent to Idexx laboratories for measurement of SDMA concentrations.[d] Additional data was recorded, including final diagnosis (if known), physical exam findings including temperature, pulse, and respiratory rate, and diet history.

Inclusion/Exclusion criteria

Final inclusion in the study required a $\geq$ 25% decline in sCr concentration between the pre and post-fluid therapy samples. Patients were excluded if they had evidence of past CKD (based on prior medical records, owner description, or imaging findings consistent with chronic kidney disease, such as small/irregular kidneys, renal cysts, increased cortical echogenicity or nephrocalcinosis on ultrasound). Patients with AKI were included in the study and analyzed with the non-AKI patients, but were also analyzed as a separate group. AKI/CKD was diagnosed based on known history of non-steroidal anti-inflammatory overdose or toxicity, *Leptospirosis* PCR[e], or clinical suspicion with compatible imaging findings (consistent with CKD) with no other apparent abnormalities on blood work.

Comparison Group

Dogs with an average age of 8.9 years that were clinically hydrated and had no known or apparent significant disease processes at the time of evaluation (e.g., diabetes mellitus, hyperadrenocorticism, neoplasia, and CKD) were enrolled to serve as a control group (n=42).

Statistical Analysis

Analyses were performed using commercial software.[f] Descriptive statistics were presented as mean (median; range) unless otherwise specified. Student's t-test was used to determine significance between the pre and post-rehydration sCr and SDMA % change and

SDMA:sCr ratios. Significance was set at $p \leq 0.05$. A post-hoc power calculation was performed using 80% power to detect a 20% difference in SDMA:sCr using the healthy dogs as true mean and the pre-treatment volume responsive azotemia patients as a comparison group with a p-value of $p \leq 0.05$.

Results

A total of 15 dogs were prospectively enrolled in the study. Of these, 9 dogs were considered partially volume responsive based on lack of return to non-azotemic status (sCr decreased > 25%, but remained > 1.5mg/dL post-fluid therapy, which is the high end of the KSU Clinical Pathology Laboratory reference interval) during the study period and six dogs had both a >25% decrease in sCr and a final sCr < 1.5mg/dL post-fluid therapy.

Dogs included in this study were an average of 9 years old (median = 9 years, range = 3 - 14 years). Breeds represented included three Labrador Retrievers, two Shorthaired Dachshunds, and one each of Pekingese mix, Australian Cattle Dog, Mastiff, Belgian Malinois, Pembroke Welsh Corgi, Doberman Pinscher, Maltese, German Shepherd, Shetland Sheepdog, and mixed breed. Listed diagnosis for the patients were diabetes mellitus (n=4), and one each of sepsis, immune-mediated hemolytic anemia, primary hyperparathyroidism, laryngeal paralysis, gastritis, trauma, possible non-steroidal anti-inflammatory (NSAID) toxicity, hypoadrenocortisism, bloody stool, possible leptospirosis, hyperadrenocorticism, and unknown diagnosis. Three patients had multiple diseases listed. Mean body weight pre-rehydration was 24.7kg (23kg; 3.15kg – 84kg). Mean body weight post-rehydration was 25.85kg (24.7kg; 3.03kg - 91.3kg). The average body weight change from pre- to post-rehydration was 3.07% (3.26%; -3.9% – 10.2%). Two patients lost weight from pre to post fluid therapy. One patient weighed 3.15kg and was re-weighed after fluid therapy at 3.03kg. The other patient weighed 14.4kg and after fluid therapy, weighed 13.9kg. Possible reasons for weight loss could include a discrepancy between scale measurements or accuracy, post-obstructive diuresis (one of the patients that lost weight had AKI), and potentially an uncooperative patient and an inaccurate scale reading due to motion. Most importantly, it is possible that inadequate fluid therapy had been administered.

USG pre-rehydration was 1.018 (1.017; 1.009 - 1.032). Notably, all but one of the dogs enrolled had limited urine-concentrating ability. One patient had a USG of 1.032 in association

with clinical dehydration. The mean pre-rehydration PCV was 40.5% (39%; 23%-67%) and the mean pre-rehydration TS was 7.1g/dL (7g/dL; 4.4g/dL - 9.5g/dL). Post-rehydration PCV and TS measurements were not available for all individuals, but both values decreased in all but one individual (who had a decrease in total solids but an increase in PCV due to receipt of a blood transfusion) after fluid therapy.

The mean pre-rehydration sCr for all dogs was 4.4mg/dL (3.6mg/dL; 2.2mg/dL – 12.4mg/dL). The mean post-rehydration sCr for dogs was 2.3mg/dL (1.7mg/dL; 0.7mg/dL – 8.4mg/dL). The mean percent decrease in sCr was 49% (50.0%; 27.3% - 75%). Pre-rehydration SDMA had a mean of 35.8ug/dL (27ug/dL; 15ug/dL - 69ug/dL). The post-rehydration SDMA had a mean of 27ug/dL (22ug/dL; 7.9ug/dL - 58ug/dL). The average percent change in SDMA was 32.8% (34%; 3.3% - 56.9%). There was a significant difference ($p=0.013$) in the percent change in sCr when compared to the percent change in serum SDMA for the pre- and post-rehydration samples in the dog group (Table 2.1).

For the healthy control group, 42 dogs were used. The average dog age was 8.9 years with a range of 7 – 14 years. Breeds represented included ten mixed breed dogs, four Cocker Spaniels, four Labrador Retrievers, three German Shepherds, three Border Collies, two Chihuahuas, two Golden Retrievers, two Boston Terriers, and one each Siberian Husky, Bichon Frise, Parsons Jack Russell Terrier, Norwich Terrier, Pembroke Welsh Corgi, Boxer, English Springer Spaniel, Dachshund, German Shorthair Pointer, Australian Shepherd, and Basenji. The mean SDMA value for these patients was 10 ug/dL (10ug/dL; 5ug/dL – 13ug/dL). All dogs had SDMA within the normal range (reference range: <14ug/dL). The standard deviation from average for SDMA was 2.23. The mean sCr value for the healthy dogs group was 0.9 mg/dL (0.9mg/dL; 0.4 – 1.2 mg/dL). The standard deviation for sCr was 0.2. All dogs had sCr within reference intervals (0.4mg/dL – 1.5mg/dL). The average SDMA:sCr for this group was 11.43 (11.25; 6.36 – 18). The standard deviation for the control group ratio was 2.77.

The SDMA:sCr value was computed for all dogs in the experimental group. The average SDMA:sCr pre-rehydration was 8.96 (7.3; 7.17 – 19.9). The post-rehydration SDMA:sCr average was 14 (11.7; 6.17 – 27.4). There was a significant difference between the pre and post rehydration SDMA:sCr values ($p=0.001$). (Table 2.1)

When the pre and post-rehydration SDMA:sCr ratio were compared to the healthy control group based on a paired t-test of unequal variance (the variance was 24.4 for the experimental

population and 7.9 for the healthy population), there was no statistically significant difference between these two groups ($p = 0.08$, $p = 0.14$, respectively). However, the difference in sCr between the pre-fluid therapy group and the control group was statistically significant ($p<0.01$), as was the difference in SDMA values for these two groups ($P<0.01$). When the post fluid-therapy group was compared to the control group, the difference between sCr and SDMA was also statistically significant ($p = 0.014$, $p< 0.01$, respectively). (Table 2.2, Table 2.3)

Five patients were diagnosed with AKI/CKD after enrollment. One had leptospirosis based on a positive PCR test on a urine sample, two had a history of NSAID ingestion, and two patients were initially suspected to have acute azotemia, but then subsequently were found to have changes on ultrasound compatible with CKD with no prior medical history of this problem. When the AKI/CKD patients (n=5) were removed from statistical analysis and the remaining 10 patients were assessed, the mean pre-fluid therapy SDMA:sCr ratio was 8.4 (7.1; 4.2 – 19.2) and was not significantly different from the SDMA:sCr ratio in the control group ($p = 0.06$). Similarly, for the post-fluid therapy (minus AKI patients) group, the mean SDMA:sCr was 14.9 and this was also not statistically significant from the control group ($p=0.09$). (Table 2.4, Table 2.5)

A post-hoc analysis of the two groups (the pre- fluid therapy group compared with the control group) showed that to detect a 20% difference in SDMA:sCr with 80% power a significance set at $p < 0.05$, 12 experimental cases would be needed. Analysis of the pre-fluid therapy group compared to the control group showed that the means were different enough (greater than 20%), so that with 80% power and a p-value of < 0.05, only 6 cases would have been needed, and 10 were included in this group (n=15-5 AKI dogs). This means that the study reported here was likely adequately powered and there was not a significant difference between the SDMA:sCr ratio between the groups.

Discussion

This study demonstrates that dogs with volume-responsive azotemia show a significantly greater decrease in sCr than the decrease in serum SDMA after rehydration with fluid therapy. Furthermore, the ratio of SDMA:sCr increased significantly after fluid therapy compared to the

pre-treatment value. No difference in the SDMA:sCr ratio was observed between the healthy control and the dehydrated patient population. Furthermore, when patients with known kidney injury (n=5) were removed from analysis, the pre-fluid and post-fluid therapy SDMA:sCr ratio was not significantly different from the control group.

SDMA has been previously used as a novel serum biomarker for estimation of GFR[19,26] and is primarily used for detection of early CKD, with some studies showing an elevation in serum SDMA with as little as 25-40% decrease in GFR.[19,25,26] The study reported here is the first study to clinically evaluate the behavior of SDMA in states of volume-responsive azotemia.

USG is the traditional test of choice for localization of azotemia (pre-renal vs. renal). However, in cases where pre-renal or volume responsive azotemia exists in the presence of another disease that causes lack of ability to produce hypersthenuric urine, the clinician is often faced with a diagnostic dilemma. In these cases, renal azotemia cannot be differentiated from pre-renal azotemia except with rehydration and subsequent measurement of creatinine in the rehydrated patient. This study shows that the SDMA:sCr ratio prior to fluid therapy would not be a helpful diagnostic test in this scenario because there was not a significant difference in this ratio between the dehydrated patients and the healthy control group. Furthermore, there was overlap between the ratios in normal and dehydrated patients.

In this study, all but one patient had volume-responsive azotemia superimposed on an inability to concentrate urine. Under these conditions, the magnitude of change of sCr post-rehydration was greater than the magnitude of change of serum SDMA and therefore volume-responsive azotemia had a greater impact on sCr than on serum SDMA. Further exploration of this ratio in dogs with pre-renal azotemia is warranted. However, based on this preliminary study, the ratio does not appear to provide insight into localization of azotemia prior to initiation of fluid therapy, unless renal azotemia can be ruled out in the individual, which may not be clinically possible prior to initiation of fluid therapy.

Potential reasons for the discrepancy between sCr and serum SDMA decreases in rehydrated patients could include underlying renal damage that was not detected by sCr. Ideally, further investigation into underlying historical CKD could have been performed in every patient in this study to rule out renal damage. Furthermore, it is clinically impossible to distinguish pre-renal azotemia from pre-renal azotemia that is severe enough to cause lasting nephron damage at initial evaluation, and this scenario may have occurred in several of the patients enrolled here. It

is also possible that once an increase in serum SDMA occurs, the SDMA is not cleared from the serum as quickly as serum creatinine. This seems less likely, as 90% of SDMA in the body is cleared via the kidneys and once rehydration has occurred, there is no known mechanism for continued increases in serum SDMA.

Ideally, the SDMA:sCr ratio would provide the clinician with a pre-treatment localization of azotemia to help determine the therapeutic plan and prognosis. Unfortunately, the SDMA:sCr ratio between healthy patients and dehydrated patients was not statistically significant based on this early pilot study. USG and subjective clinical assessment of dehydration, plus evaluation of the patient for concurrent disease that could limit urine concentrating ability remain the diagnostic options for assessing pre-renal versus renal azotemia prior to fluid therapy in states where the azotemia is super-imposed on an inability to concentrate urine due to other disease.

A significant difference also did not exist between any of the groups when the AKI patients were excluded from analysis. This means that the SDMA:sCr ratio is unlikely to benefit prediction of volume-responsive azotemia. The SDMA:sCr ratio was lower in patients with dehydration and no AKI than in the control group and this was nearly significant ($p=0.06$). The SDMA and sCr values between these groups were significant, but this offers no additional pre-treatment insight into localization of azotemia.

There were several limitations to the study reported here. First, imaging was not done in every patient to help rule out underlying CKD. It is possible that the sCr returned to normal in dehydrated, early stage CKD patients, and so these patients were not recognized as having CKD. It is also not possible to evaluate the pre-treatment renal damage associated with severe and prolonged dehydration, and this factor could cause variability in SDMA and sCr. Secondly, there was no objective measurement for hydration in this study. Measurement of hydration status in the veterinary patient is clinically subjective. Bioimpedance analysis has been attempted in veterinary medicine,[32,33,34,35] but due to the labor-intensive and time-consuming nature of this test, it is rarely performed in routine clinical medicine. Instead, we relied on subjective assessments of rehydration (skin tent, capillary refill time, etc.) and objective measurements of hydration status (body weight change, PCV/TS change) in this study. It is also possible that the volume of fluid therapy intended to rehydrate these dogs was inadequate, as all dogs receiving fluid therapy were estimated at greater than or equal to 5% dehydrated and the mean body weight gain was only 3%, rendering the fluid therapy inadequate in some cases.

Many of the dogs in this study received continued fluid therapy, and it could have been potentially more useful to measure the SDMA:sCr ratios in patients that had gained back their total estimated body weight, or at least greater than 5%, rather than at a point where sCr had decreased by 25%.

Conclusions

Both sCr and SDMA decreased in response to rehydration in patients with volume-responsive azotemia. However, the magnitude of serum SDMA change was significantly less than the magnitude of sCr change in patients with volume responsive azotemia superimposed on an inability to concentrate urine. The SDMA:sCr ratio was significantly higher after volume rehydration. However, no significant difference was found between pre or post-treatment patients and the control group. Therefore, the SDMA:sCr is considered not useful for the differentiation of pre-renal and renal azotemia prior to initiation of fluid therapy based on this early pilot study. Further studies are needed to adequately assess hydration status in patients with pre-renal azotemia and compare SDMA and sCr changes in these individuals.

Chapter 3 - Longitudinal Evaluation of Serum SDMA and SCr in Dogs and Cats

Introduction

CKD is a common problem and major cause of morbidity and mortality in older dogs and cats. Although CKD is irreversible and often progressive, feeding a diet formulated for long-term maintenance and nutrition for CKD patients, along with an enteric phosphate binder has been shown to improve survival in dogs and cats with CKD.[16,37,38,39,40,41,42] It is a logical, but unproven, hypothesis that early diagnosis of CKD and early initiation of renoprotective treatments (e.g., phosphate binders, angiotensin-converting enzyme inhibitors (ACEi), anti-hypertensive therapies, etc.) will be associated with improved outcomes (Figure 1.2).[16] Early diagnosis of CKD is however somewhat elusive. Early clinical signs tend to be mild and non-specific and standard clinicopathologic tests remain normal until greater than ¾ of the nephron mass is lost. Plasma clearance techniques to estimate GFR are readily available and have increased sensitivity for early diagnosis of CKD but are cumbersome and remain underutilized in clinical practice. Longitudinal assessment of sCr has also been shown to have increased sensitivity for early diagnosis of CKD compared with a single sCr determination but longitudinal data is not always available and decreases in patient muscle mass can confound interpretation of sCr.[16,29] Therefore, research has focused on new serum biomarkers that may aid in early detection and thus potentially early treatment of CKD in both dogs and cats.

Symmetric dimethylarginine (SDMA) has emerged as a serum biomarker for early detection of CKD in veterinary medicine. Briefly, SDMA was first identified in 1970 by Kakimoto and Akazawa.[1] It is now known that SDMA is a byproduct of cellular protein metabolism, specifically the methylation of arginine residues and their subsequent release during proteolysis.[1] SDMA appears to be a relatively biologically inert molecule, however, it may function as an indirect inhibitor of nitric oxide synthase.[4,6,7] The potential use of serum SDMA as a marker for evaluation of GFR in human and veterinary medicine has been extensively studied.[7,19,25,26,27] SDMA is not protein bound in plasma, it is freely filtered by the glomerulus, and is not secreted or re-absorbed by the tubules. SDMA is eliminated from the body primarily

via renal excretion (>90%), making it a possible candidate biomarker for evaluation of GFR.[7]

A commercial assay for measurement of canine and feline SDMA is now available to practitioners. Previous studies have demonstrated that SDMA correlates well with GFR and sCr in dogs and cats.[19,24,26,27] In a study by Hall et al in 2014, SDMA had a higher sensitivity for detection of early CKD (100%) than sCr (17%), but a lower specificity (91%) than sCr (100%). Braff et al in 2014 determined that SDMA and GFR are closely correlated in cats (R = -0.82). Similarly, SDMA and sCr were also closely correlated, suggesting that, in this study, SDMA performed at least equally as well as sCr as an endogenous marker of GFR.[27]

SDMA has several possible advantages over measurement of sCr for early detection of CKD. First, sCr usually remains within reference ranges until approximately 75% nephron loss has occurred. Conversely, SDMA has been shown to detect CKD when 40% of nephron loss has occurred, allowing for earlier detection and intervention.[19] It could be hypothesized that SDMA potentially has a more linear correlation with GFR, whereas sCr has a more curvilinear relationship to GFR. With early nephron loss, GFR can decrease markedly with very little increase in the sCr concentrations. SDMA has been shown to detect decreases in GFR in some cases with as low as a 25% decrease in GFR.[19,26] Secondly, sCr is influenced by body condition of lean muscle mass, which can be markedly decreased in patients with CKD, resulting in an overall lower sCr concentration that may not truly reflect the decrease in GFR.[16,30,31] sCr concentrations, but not SDMA, were influenced by lean body mass in a study of dogs by Hall *et al* in 2015.[29]

The objective of the current study was to further compare sensitivity and specificity of serum concentrations of SDMA and creatinine for detection of early CKD in a prospective, longitudinal study of older dogs and cats.

Materials and Methods

Animals

Healthy dogs and cats, primarily owned by first-year veterinary students and faculty and staff from the College of Veterinary Medicine at Kansas State University were recruited for this prospective, longitudinal study. The Institutional Animal Care and Use Committee of Kansas

State University approved the study and written owner consent was obtained prior to patient entry into the study.

Inclusion/Exclusion Criteria

Dogs and cats had to be ≥ 7 years of age and were apparently disease-free at the time of enrollment (e.g., no known diabetes mellitus, hyperadrenocorticism, neoplasia, and CKD). Cats and dogs were excluded if a fractious temperament prevented frequent handling or multiple sample collections. Dogs and cats were also excluded if the owners would not be available for periodic examination over the intended 4-year study period.

Study Design

A four-year, prospective, observational study was conducted. Each dog and cat was evaluated biannually, at approximately 6-month intervals. At each evaluation, the patient had a complete physical examination and historical information was collected and recorded, including current diet, environment, past medical history, current medications, and any other pertinent information.

At each evaluation, systolic blood pressure was measured at every time point by Doppler methodology.[g] Clinicopathologic tests including a complete blood count (CBC), biochemistry profile, serum total thyroxine, urinalysis with culture, urine protein: creatinine ratio (UPC), and SDMA were measured at every time point.[d] Over the course of the study, if history, physical examination findings or the clinicopathologic data suggested a possible decrease in renal function (including a history of polyuria/polydipsia, abnormal kidney palpation, or increases in sCr or SDMA without hypersthenuria, persistent proteinuria, or persistent decreases in urine specific gravity), plasma clearance of iohexol and abdominal ultrasound examinations were added to the biannual evaluations.

Blood and Urine Sample Collection and Analysis

Blood samples were collected from the jugular or lateral or medial saphenous veins. All urine samples were collected by cystocentesis. All laboratory evaluations were performed by

Idexx laboratories.[d] Blood and urine samples were collected and serum harvested Monday through Thursday and shipped overnight on dry ice to be analyzed within 24 hours of collection. Blood for serum biochemistry was stored in a red-top plastic serum tube and was centrifuged with the serum harvested prior to shipping, complete blood count was performed on blood that had been stored and shipped in ethylenediaminetetraacetic acid (EDTA) tubes.

Symmetric Dimethylarginine

Symmetric dimethylarginine concentrations were determined by Idexx laboratories using high-performance liquid chromatography mass spectrometry (LC-MS) methodology, as previously reported.[d,19,20] The normal reference interval for SDMA in healthy cats and dogs was previously determined to be <14 μg/dL.

CBC/Biochemistry Profile

The complete blood count and biochemistry profiles were performed using standard operating procedures by Idexx laboratories.[d] Each CBC was reviewed by a technical specialist for accuracy and presence of organisms or abnormal cells. Reference ranges for both CBC and biochemistry profiles were previously established using healthy dogs and cats.

Urinalysis, Urine Culture, and UPC

Urine specific gravity was determined using a refractometer. Hypersthenuria was defined as greater than 1.030 for dogs and greater than 1.035 for cats. All laboratory protocols were followed, as previously reported.[d] Each urinalysis had a sediment examination that was reviewed by a laboratory technician.[d] Approximately 0.5 mL of urine was saved from each urine sample and refrigerated until submitted for aerobic culture and sensitivity. Urine protein concentrations were determined using urine supernatant. Urine protein was quantified using the benzethonium reaction method and urine creatinine was quantified using the buffered Jaffe reaction, both with an automated chemistry analyzer.[d] The UPC was calculated from this data for each urine sample.

Histopathology

Complete necropsies were performed when possible when dogs or cats died or were euthanized. Histopathologic samples were obtained from the kidney and any other grossly affected organs or organs of interest based on the cause of death and clinical signs/laboratory tests. Histopathologic analysis was performed with standard sample processing and light microscopy at the Kansas State University Veterinary Diagnostic Laboratory.[e]

Blood Pressure Measurement

Systolic blood pressure measurements were obtained via an ultrasonic Doppler[g] monitor after the dogs and cats were acclimated to the hospital environment but prior to physical examination or specimen collection. Dogs and cats were placed in lateral recumbency and the up forelimb was prepared for pressure measurement by slipping the hair over the common digital branch of the radial artery. An inflatable cuff with a width approximately 35-40% the circumference of the leg was placed directly below the elbow and secured. The cuff was briefly inflated to a pressure of approximately 240mmHg to stop arterial flow and was then deflated gradually until an audible pulse signal, representing systolic pressure, returned. The systolic blood pressure recorded represented the mean of 3-4 separate determinations. At subsequent evaluations, blood pressure measurements were obtained using the same body position, limb, and cuff size.

Determination of GFR

A single intravenous injection of iohexol (300 mg/kg), was administered via a cephalic or saphenous vein. Three blood samples were collected at 2, 3, and 4 hours after injection from the jugular vein or opposite saphenous vein. Serum concentrations of iohexol were measured by a commercial laboratory[h] using an inductively coupled plasma-atomic emission spectroscopy (ICP-AES) method. GFR was estimated from calculations made using a one-compartment model for serum iohexol clearance. Median (range) GFR for normal healthy cats and dogs was determined by the laboratory and was 1.94 mL/min/kg (range, 1.15 – 2.73 ml/min/kg) and 5.48 ml/min/kg (range, 2.89 – 8.07 ml/min/kg), respectively. The percentage above or below the population average median for dogs and cats (previously determined) was also reported.

CKD Definition

For the purposes of this study, CKD was defined/diagnosed in dogs and cats by one or more of the following abnormalities:

1. Renal changes compatible with CKD observed by ultrasound examination (e.g., increased cortical echogenicity, loss of the corticomedullary junction, cortical infarcts, irregular renal contour, nephrolithiasis)
2. Renal proteinuria (UPC > 0.5 in dogs and > 0.4 in cats) that was present on 2 or more urine evaluations with normal urine sediment examinations.
3. A decrease in GFR $\geq$ 40% when the physical examination showed no evidence of dehydration and the patient did not exhibit hypersthenuria.
4. Renal histologic changes compatible with CKD at necropsy examination.

Statistical Analysis

Statistical analysis was performed using standard statistical software[f,i]. Data are reported as mean (median; range) unless otherwise indicated. Sensitivity and specificity were calculated and McNemar's test was used to determine equality of marginal frequencies of the sensitivity and specificity data. A chi-square test was used to determine significance. A Fisher's exact test was used to determine significance of contingency groups. The P-value was set at <0.05.

Dogs and cats with CKD were identified according to above criteria over the four-year period of time. Dogs and cats that were prospectively enrolled in the study that did not develop CKD were used as an age-matched comparison group.

Results

Dogs - A total of 43 dogs were enrolled in the study. At the time of enrollment, the average dog age was 8.9 years with a range of 7 – 14 years. Breeds represented included ten mixed breed dogs, four Cocker Spaniels, four Labrador Retrievers, three German Shepherds, three Border

Collies, two Chihuahuas, two Golden Retrievers, two Boston Terriers, and one each Siberian Husky, Bichon Frise, Parsons Jack Russell Terrier, Norwich Terrier, Pembroke Welsh Corgi, Boxer, English Springer Spaniel, Dachshund, German Shorthair Pointer, Australian Shepherd, and Basenji. Twelve patients died or were euthanized during (n=9) or shortly after the study period (n=3) and more than half of the eight samples had been collected for patients that died during the study period. Necropsy was performed in nine of 12 dogs that died or were euthanized. Cause of death was determined to be neoplasia (hemangiosarcoma (n=3), gastric adenocarcinoma (n=2), hepatocellular carcinoma (n=2), bronchoalveolar carcinoma, mesothelioma, chemodectoma, pulmonary adenocarcinoma, melanoma, gastrointestinal stromal cell tumor, and nasal adenocarcinoma) in 11 dogs, laryngeal paralysis and cervical intervertebral disc disease in one dog, and non-suppurative encephalitis in 1 dog (some dogs had multiple neoplasms or causes for euthanasia). The listed reason for euthanasia for the 3 dogs that were euthanized without necropsy were 1) severe arthritis, 2) a bladder wall mass and medial iliac lymphadenopathy and inability to urinate, and 3) severe cervical disc disease.

CKD was documented in 23 dogs (53%) by US abnormalities (n=13), decreased GFR (> 40% reduction) (n=13), persistent renal proteinuria (UPC $\geq$ 0.5) (n=6), or renal histology (n=6). Twelve dogs had multiple abnormalities. (Figure 3.1)

9 of 23 CKD dogs had increased SDMA ($\geq$ 14µg/dl) without hypersthenuria concurrent or subsequent to CKD diagnosis. One dog had a single (endpoint) increased SDMA and 8 had persistent/multiple SDMA increases. Conversely, only 2 of the 23 dogs had increased sCr (> 1.8mg/dl upper end of Idexx laboratory reference interval) at any point and both of these dogs had concurrent/prior SDMA increases. In the 13 dogs with decreased GFR (40-80% reduction from median baseline), sCr and SDMA were increased in 1 and 7 dogs, respectively. There were no persistent increases in SDMA without hypersthenuria in dogs without CKD. There were also no instances when SDMA was within a normal range and sCr was elevated. (Figure 3.2) Increased SDMA without hypersthenuria had 39% sensitivity and 100% specificity for CKD whereas increased sCr without hypersthenuria had 9% sensitivity but 100% specificity, which was significantly different (p=0.024).

Of the dogs with CKD, five of 23 patients (22%) had persistent hypertension, classified as moderate (n=3) with a persistent systolic blood pressure of greater than or equal to 160mmHg, or severe (n=2) with a persistent systolic blood pressure of greater than or equal to 180mmHg.

Diet was recorded as part of each visit. Of the 43 dogs enrolled in the study, all dogs were fed commercially available diets. Fourteen were reported to be on diet formulated for senior dogs, 16 were fed diets formulated to support joint health, 10 were fed a maintenance diet, 4 were fed a diet formulated to support gastrointestinal health, three were fed weight loss diets, 2 were fed novel antigen diets, and 1 dog (who was not diabetic) was fed a diet formulated for diabetic dogs. Some dogs were fed multiple diets throughout the 4-year study period. Six dogs in the CKD group were transitioned to a commercial renal diet throughout the course of the study.

17 of 43 dogs (n=11 males, n=6 females) developed a urinary tract infection by quantitative culture from a urine sample that was collected by cystocentesis using aseptic techniques. Only 4 dogs were reported to have clinical signs consistent with UTI at the time of UTI diagnosis. The other 13 dogs were either asymptomatic or signs were not reported. Organisms represented included *P. mirabilis, E. coli, Enterococcus spp., P. aeruginosa, Candida albicans, S. pseudointermedius,* and *K. pneumoniae.* Dogs were treated with oral antibiotics (based on a sensitivity profile). All dogs treated with antibiotics had subsequent resolution of the bacterial growth on urine culture at the next time point, but were evaluated prior to the six month recheck for repeat culture and sensitivity to ensure their UTI had resolved. Of the 17 dogs that developed urinary tract infections, 13 also developed CKD (76%).

Of the dogs in the CKD group (n=23), 13 of the dogs lost weight (56.5%) and 10 of the dogs gained weight (43.4%). In the non-CKD group (n=20), 13 dogs lost weight (65%) and 7 dogs gained weight (35%). There was no significant difference between the number of dogs that lost weight in the CKD group compared to the non-CKD group and there was no significant difference between the two groups regarding kilograms lost. Of the dogs in the CKD group that lost weight, the average amount of weight lost was 3.36kg (median: 2.11kg, range: 0.67-14.4kg). The average body weight loss percent was 17.5% (13.5%; 3.9% - 40.3%). The patient who lost 14.4kg (40.3% body weight) died of a gastrointestinal stromal cell tumor and hepatocellular carcinoma, which could partly explain the profound weight loss in this patient. Of the dogs in the non-CKD group that lost weight, the average amount of weight lost was 1.74kg (median: 0.82kg, range: 0.3-6.8kg). The dog that lost 6.8kg died of a gastric adenocarcinoma. The percent of weight lost in the non-CKD group was an average of 6.4% (4.8%; 2.7% - 15.3%). While the actual number of kilograms lost and the number of dogs that lost weight was not

statistically significant between the two groups, the percentage of weight loss was significantly greater in the CKD group than in the non-CKD group (p=0.0043), based on a paired t-test. Of the dogs that had CKD and weight loss, 46% (n=6) had neoplasia that was identified during the course of the study. Only two dogs (15%) that did not have CKD and lost weight had neoplasia. Of the dogs that had CKD and weight loss (n=13), 8 dogs (61.5%) had normal creatinine and increased SDMA.

Of the dogs with CKD (n=23), only 4 (17.4%) had an increase in sCr of greater than 0.3mg/dL over the course of the study. In these four patients, SDMA was persistently elevated in 3 of them (75%).

Some dogs developed concurrent chronic diseases that were diagnosed antemortem throughout the course of the study. In the CKD group, 15/23 dogs developed concurrent chronic disease. Diagnoses included a heart murmur (n=7), arthritis (n=2), diabetes mellitus (n=2), skin allergies (n=2), and hypothyroidism (n=2). In the non-CKD group, 8/20 patients developed concurrent chronic disease. Diagnoses included a heart murmur (n=1), arthritis (n=3), skin allergies (n=1), Arrhythmogenic right ventricular cardiomyopathy (n=1), neurologic manifestations suspected to be due to a cerebral vascular accident (n=1), and a thyroid mass (n=1). There was no significant difference between CKD and non-CKD groups regarding development of concurrent disease (p=0.131)

Cats – A total of 33 cats were enrolled in the study. At the time of enrollment, the average age of cats was 9.4 years with a range of 7-15 years. Twenty-one cats were listed as domestic shorthair cats, 9 were domestic longhair cats, 2 were Siamese and 1 was a Ragdoll. Seven patients were euthanized during or within 5 months after completion of the study period. Listed reasons for euthanasia were seizures, ulcerative skin lesions, CKD with poor quality of life, pancreatitis, and aortic thromboembolism.

CKD was documented in 13 (39%) cats by renal US abnormalities (n=10), decreased GFR (> 40% reduction) (n=1), persistent renal proteinuria (UPC $\geq$ 0.4) (n=3), or renal histology (n=3). Four cats had multiple abnormalities. (Figure 3.3)

Seven of the 13 CKD cats had increased SDMA ($\geq$ 14µg/dl) without hypersthenuria concurrent or subsequent to CKD diagnosis. Two cats had a single (endpoint) increased SDMA and 5 had persistent/multiple SDMA increases. Conversely, 1 of the 13 CKD cats had increased sCr (>2.3mg/dl, Idexx Laboratory upper end of reference interval) and this cat had a concurrent

increase in SDMA. In the 1 cat with decreased GFR (49.4% below median), sCr was within referenace intervals (1.8mg/dL) and SDMA was increased (14µg/dL). There was 1 cat with multiple increases in SDMA without hypersthenuria without other evidence of CKD. There were no instances of increased sCr and normal SDMA. (Figure 3.4) Increased SDMA without hypersthenuria had 54% sensitivity and 95% specificity for CKD whereas increased sCr without hypersthenuria had 8% sensitivity and 100% specificity (p=0.042). None of the 13 cats with CKD had persistent elevations in blood pressure (>160mmHg).

Most cats were fed more than one commercially available diet during the study. Of the 33 cats enrolled, 24 (73%) were fed diets available by prescription only. Diets fed included diets formulated for overweight management, hairball control, easily digestible diets, and Purina DM™.

Four of the 33 cats enrolled in the study had one or more positive urine culture results during the study period (n=3 females, n=1 male). All cats with urinary tract infections also had CKD (100%). Bacteria grown on culture medium included *Enterobacter sp., Pseudomonas sp., Klebsiella oxytoca,* and *E. coli*. Cats were treated with appropriate oral antibiotics (based on sensitivity profiles) at the discretion of the attending clinician. Follow-up cultures were negative in three and unavailable in one cat due to study endpoint.

Of the 13 cats with CKD, seven lost weight (53.8%) and 6 had no change or gained weight (46.2%). The average amount of weight lost was 0.94kg (median: 0.67kg, range: 0.52kg-1.71kg). In the Non-CKD group, 5 (25%) lost weight and 15 (75%) gained weight or had no change. The average amount of weight lost was 0.8kg (median: 0.7kg, range: 0.42kg-1.28kg). There was no significant difference between these groups (p=0.142). In the CKD cat group, the percent body weight loss averaged 15.36% (14.4%; 11.6% - 23.9%). In the non-CKD group, the mean body weight lost was 17.32% (18.2%; 7.7% - 23%). Of the CKD cats that lost weight (n=7), 6 cats (85.7%) had elevated SDMA in the face of normal sCr (< 2.1mg/dL).

In the CKD group (n=13), five cats had a trending increase in sCr (>0.3mg/dL) over time. Of these cats, four of them (80%) had increased SDMA.

Discussion

This study is the first prospective, longitudinal evaluation of older dogs and cats that compared sensitivity and specificity of serum SDMA and sCr for detection of early CKD. Our results show that persistently (or endpoint) increased serum SDMA without hypersthenuria is a specific renal biomarker for detection of CKD and is more sensitive than sCr. Serum SDMA was measured over time in this study. We considered only persistent or endpoint increases in serum SDMA to be predictive of CKD. In dogs, increased SDMA without hypersthenuria was a significantly more sensitive indicator of CKD than sCr (39% versus 9%, respectively). The specificity of SDMA was equal to that of sCr in dogs (100%). In cats, SDMA had a slightly lower specificity (95%) than sCr (100%). Similarly, in cats, serum SDMA without hypersthenuria was significantly more sensitive than sCr for detection of CKD (54% versus 9%, respectively). Clinically, persistent elevations in SDMA are a better predictor of CKD than sCr because the sensitivity is increased. Clinicians should evaluate this test carefully. It is not intended to be a one-time predictor of CKD and is also not intended for use in sick or dehydrated dogs and cats. As with any test, interpretation in light of other clinical signs compatible with CKD is recommended. If no signs are present, documentation of persistent increases in SDMA should result in increased monitoring of that individual over time.

It is widely known that creatinine is produced from muscle turnover, and can be falsely decreased in patients with decreased muscle mass, which often occurs in patients with CKD.[16,31,43] Serum SDMA concentrations do not appear to be impacted by lean body mass in healthy beagles.[29] In the Hall 2015 study, lean body mass was measured using dual-energy x-ray absorptiometry and sCr and SDMA were also measured over a six month period.[29] Changes in sCr were significantly correlated with changes in lean body mass, whereas SDMA was not. In our study, of the CKD dogs that lost weight, 61% of them had normal sCr with increased SDMA. This was also true in CKD cats in this study, as 75% of the cats with CKD that lost weight had elevated serum SDMA and normal sCr. Because weight loss and loss of lean body mass is a common problem in dogs and cats with CKD, sCr can lag behind in prediction of CKD and can (in some cases) dramatically underestimate the true decrease in GFR. SDMA appears to be unaffected by muscle mass, although the study reported here did not incorporate dual-energy x-

ray absorptiometry. Further prospective studies are needed in CKD patients to determine how decreased muscle mass affects SDMA over time.

A trend of progressive increased sCr over time, even if the sCr level remains within reference intervals may be compatible with early non-azotemic CKD.[17] An increase in sCr of greater than 0.3mg/dL over time in the face of non-hypersthenuria has been proposed as a potential marker for diagnosis of early CKD.[17] Similarly, persistent increases in SDMA in the presence of non-hypersthenuria should trigger suspicion for CKD and increased monitoring is recommended. Longitudinal increases in sCr (>0.3mg/dl) without hypersthenuria was only valuable for documenting early CKD in 17.4% of the dogs, and of these, 75% of them had increased serum SDMA. In the feline group, 38% of the cats had longitudinal increase in sCr without hypersthenuria and, of these, 80% of them had elevated SDMA.

CKD was evaluated over the four-year time period in this study and was diagnosed based on GFR and markers of IRIS Stage 1 CKD. However, one limitation of this study is that GFR, ultrasound, and renal histology were not performed in every patient. This may have introduced some bias and some patients may have had unrecognized CKD if it had not progressed to a point where changes that triggered ultrasound and GFR measurements were not present. However, our study more closely describes the situation that would occur in clinical practice, where it is uncommon to obtain GFR, abdominal imaging, and histopathology on an otherwise healthy patient with unremarkable clinicopathologic findings and urinalysis.

Another important consideration in the design of this study was that the diagnosis of CKD was based on a combination of imaging findings, proteinuria, and histology, which are not direct markers of GFR. GFR measurements were not performed in every case. In turn, we sought to measure the sensitivity and specificity of SDMA, which is intended to be used as a GFR biomarker, not an imaging marker, a marker of proteinuria, or a histology biomarker. This introduces some interesting interpretation into the study because the definition of CKD may not reflect actual changes in GFR for the patients enrolled. Because of the prospective nature of this study, we used standard evaluations for defining and diagnosing CKD and, to the best of our ability, identified this disease accurately in every patient. However, for example, a patient with bilateral nephrocalcinosis has CKD by definition (CKD is defined as a structural and/or functional abnormality of one or both kidneys that has been continuously present for three months or longer), but may have minimal GFR impairment, meaning that SDMA would, also by

definition (as a GFR biomarker) not be elevated in this individual. Therefore, while SDMA is a more sensitive biomarker than sCr, it may not actually predict renal histologic or ultrasonographic changes that are compatible with CKD. SDMA remains more sensitive than sCr, but comparison of SDMA to ultrasonographic and histopathologic renal changes is questionable. Again, GFR measurements on every patient in the study would have been more ideal for assessment of SDMA than the CKD definitions used here.

Hypertension, defined as persistent elevation in systolic blood pressure (>160mmHg for moderate hypertension in dogs and cats and >180mmHg for severe hypertension in dogs and cats) by Doppler technique, was documented in 22% of the CKD dogs but none of the CKD cats in this study. The low prevalence of hypertension in the CKD dog and cats may be associated with early detection of CKD that was not severe enough to cause high blood pressure.

Diet changes occurred during the study period in almost all of the patients enrolled. This could be due to the fact that most enrolled patients were owned by veterinary students and staff at the KSU-VHC and discounted or free pet food available through feeding programs may have influenced diet choices. Approximately half of the dogs and ¾ of the cats in this study were fed a diet available by prescription only. Patients with CKD were transitioned to a renal diet (or other diet, if needed).

In this study, 100% of the cats that developed urinary tract infections also had CKD. In the feline group, 75% of the cats that developed UTIs were female. Conversely, 76% of the dogs that developed urinary tract infections had CKD and in the dog group that developed UTIs, only 35% were female. Urine culture remains an important diagnostic tool for patients with CKD because of the potential for development of urinary tract infections that may ascend and result in pyelonephritis.

A criticism of previous studies regarding sensitivity and specificity of SDMA compared to sCr is that the laboratory reference intervals used for diagnosis of azotemia are variable. In our study, using the lower value of >1.4 mg/dL in dogs and greater than 1.6mg/dL (e.g., IRIS Stage 2 guidelines) in cats would not alter the number of dogs and cats with CKD, nor would it affect the sensitivity and specificity of creatinine. The results using the IRIS stage 2 cut-off versus the laboratory reference interval in terms of sensitivity and specificity are the same.

Conclusion

This study demonstrates that over a four-year period of time in which otherwise healthy patients developed naturally occurring CKD, serum SDMA concentration was a more sensitive marker for CKD compared with sCr concentration. Assessment of serum SDMA concentrations in conjunction with urine specific gravity may aid in earlier diagnosis and recognition and CKD and therefore, earlier initiation of therapy and serial monitoring.

Tables and Figures

Table 1.1 IRIS staging of CKD in dogs

Stage	sCr (mg/dL)	Comments	Sub-stage by UPC	Sub-staging by systolic blood pressure
I	<1.4	Non-azotemic with some other abnormality present	<0.2 (non-proteinuric) 0.2-0.5 (borderline proteinuric) >0.5 (proteinuric)	<150mmHg (minimal risk for target organ damage - TOD) 150-159 (low risk for TOD) 160-179 (moderate risk for TOD) >180 (high risk for TOD)
II	1.4-2.0	Mild renal azotemia		
III	2.1-5.0	Moderate renal azotemia		
IV	>5.0	Severe renal azotemia		

Table 1.2 IRIS staging of CKD in cats

Stage	sCr (mg/dL)	Comments	Sub-stage by UPC	Sub-staging by systolic blood pressure
I	<1.6	Non-azotemic with some other abnormality present	<0.2 (non-proteinuric) 0.2-0.4 (borderline proteinuric) >0.4 (proteinuric)	<150mmHg (minimal risk for target organ damage - TOD) 150-159 (low risk for TOD) 160-179 (moderate risk for TOD) >180 (high risk for TOD)
II	1.6-2.8	Mild renal azotemia		
III	2.8-5.0	Moderate renal azotemia		
IV	>5.0	Severe renal azotemia		

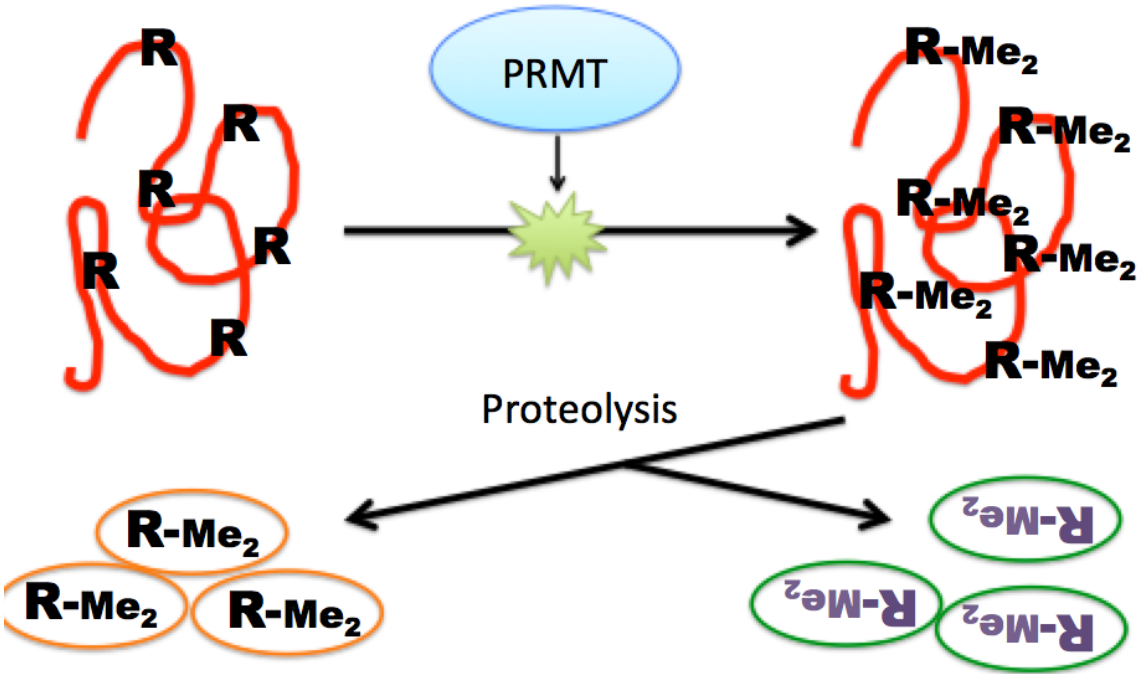


Figure 1.1 SDMA Production – A protein, specifically the arginine residue, is modified by PRMT and becomes methylated. It is then broken down by proteolysis into symmetric and symmetric dimethylarginine. (PRMT = Protein arginine N-methyltransferase, R = arginine residue, R-Me_2 = Symmetric and asymmetric dimethylarginine)

Figure 1.2 Potential effects of early diagnosis and treatment of CKD (credit: Dr. Greg Grauer)

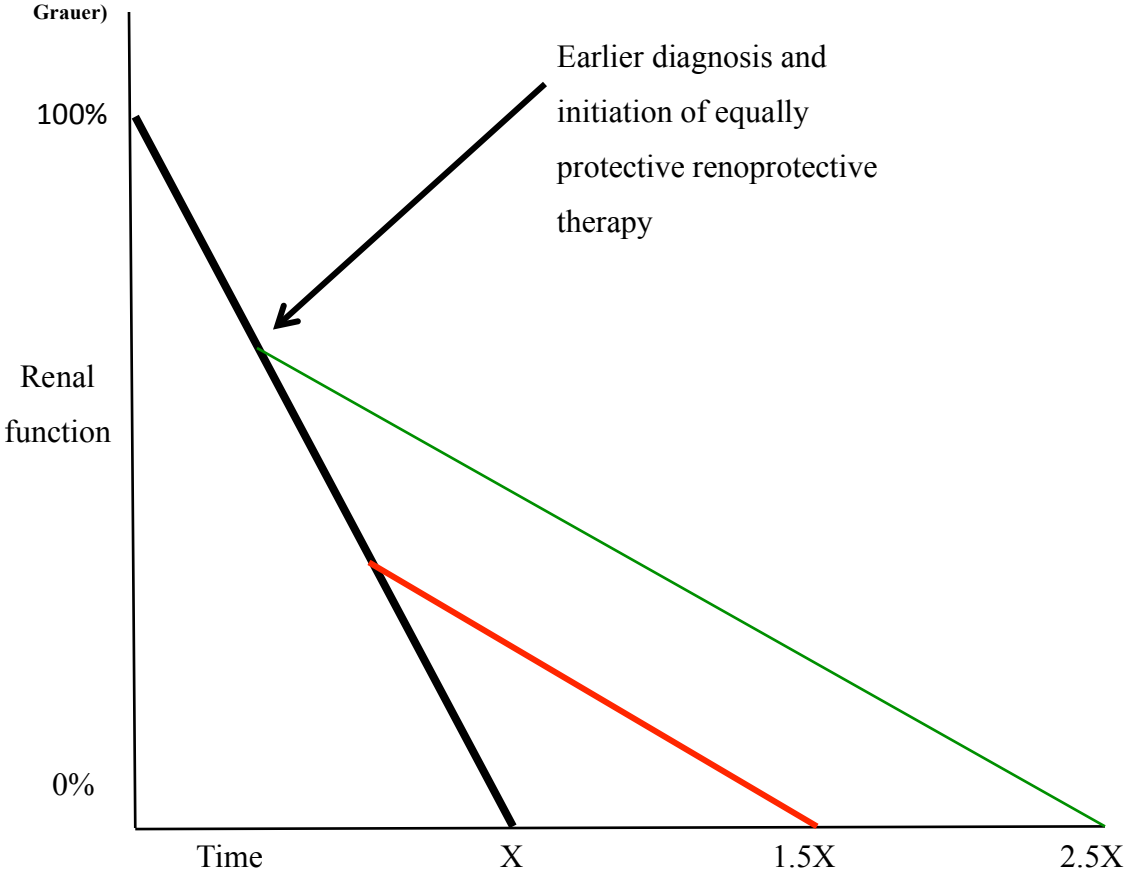


Table 2.1 Pre and post fluid therapy comparisons for BW, sCr, SDMA, and SDMA:sCr ratio

	Pre-fluid therapy	Post-fluid therapy	% change
Body weight (kg)	24.7 (3.15 – 84)	25.9 (3.03 – 91.3)	3.1% (-3.9 – 10.2%) (p = 0.04)
sCr (mg/dL)	4.4 (2.2 – 12.4)	2.3 (0.7 – 8.4)	- 49% (27 – 75) (p < 0.0001)
SDMA (ug/dL)	35.8 (15 – 69)	27 (7.9 – 58)	- 32.8% (3.3 – 56.9%) (p = 0.06)
SDMA:sCr	8.96 (7.2 – 19.9)	14 (6.2 – 27.4)	p = 0.001

Table 2.2 sCr, SDMA, and SDMA:sCr ratio in pre-fluid therapy versus controls

	Pre-fluid therapy	Control Group	P-value
sCr (mg/dL)	4.4 (2.2 – 12.4)	0.9 (0.4 – 1.2)	< 0.01
SDMA (ug/dL)	35.8 (15 – 69)	10 (5 – 13)	< 0.01
SDMA:sCr	8.96 (7.2 – 19.9)	11.4 (6.4 – 18)	0.08

Table 2.3 sCr, SDMA, and SDMA:sCr ratio in post-fluid therapy versus controls

	Post-fluid therapy	Control Group	P-value
sCr (mg/dL)	2.3 (0.7 – 8.4)	0.9 (0.4 – 1.2)	0.014
SDMA (ug/dL)	27 (7.9 – 58)	10 (5 – 13)	< 0.01
SDMA:sCr	14 (6.2 – 27.4)	11.4 (6.4 – 18)	0.14

Table 2.4 sCr, SDMA, and SDMA:sCr ratio in pre-fluid therapy (minus AKI/CKD) versus controls

	Pre-fluid therapy (minus AKI/CKD)	Control Group	P-value
sCr (mg/dL)	8.4 (4.2 – 19.2)	0.9 (0.4 – 1.2)	< 0.01
SDMA (ug/dL)	27 (15 – 69)	10 (5 – 13)	< 0.01
SDMA:sCr	8.4 (4.2 – 19.2)	11.4 (6.4 – 18)	0.06

Table 2.5 sCr, SDMA, and SDMA:sCr ratio in post-fluid therapy (minus AKI/CKD) versus controls

	Post-fluid therapy (minus AKI/CKD)	Control Group	P-value
sCr (mg/dL)	1.7 (0.7 – 3.4)	0.9 (0.4 – 1.2)	< 0.01
SDMA (ug/dL)	24.7 (7.9 – 58)	10 (5 – 13)	< 0.01
SDMA:sCr	14.9 (7.7 – 27.4)	11.4 (6.4 – 18)	0.09

Figure 3.1 Abnormalities in CKD dogs

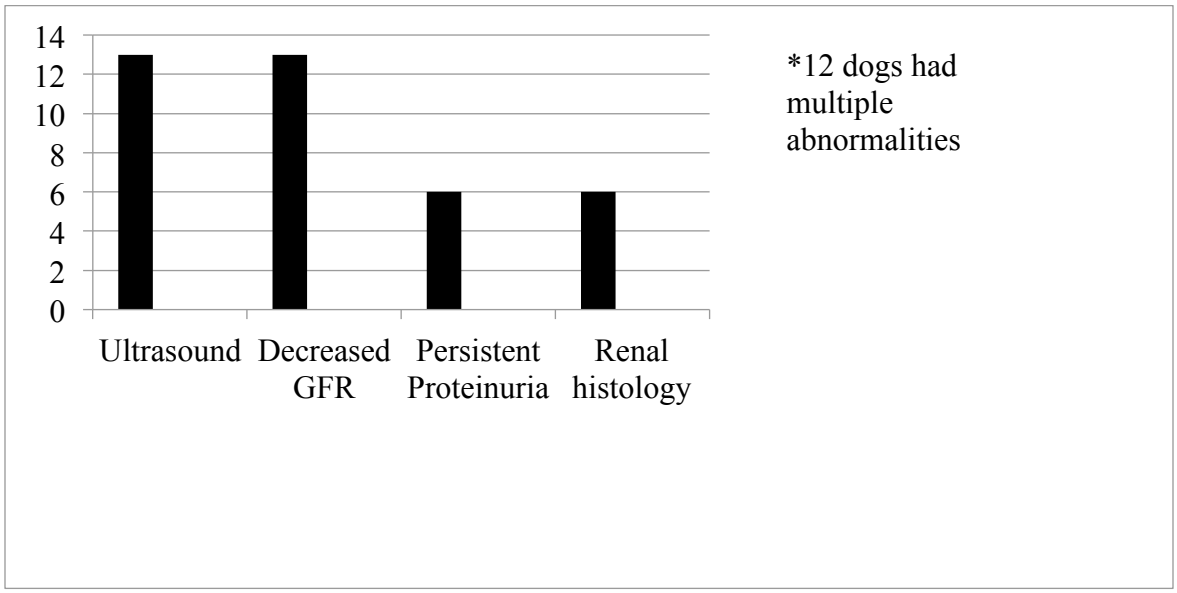


Figure 3.2 sCR versus SDMA increases in CKD and non-CKD dogs

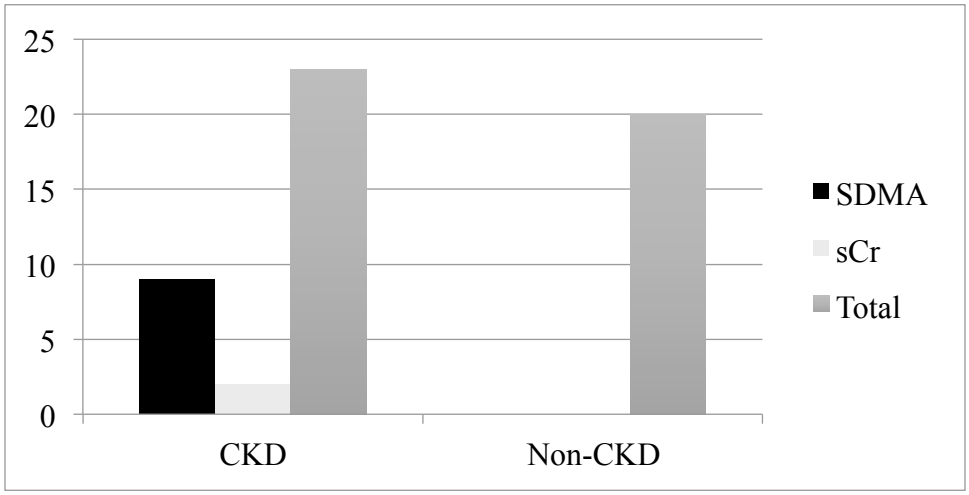


Figure 3.3 Abnormalities in CKD cats

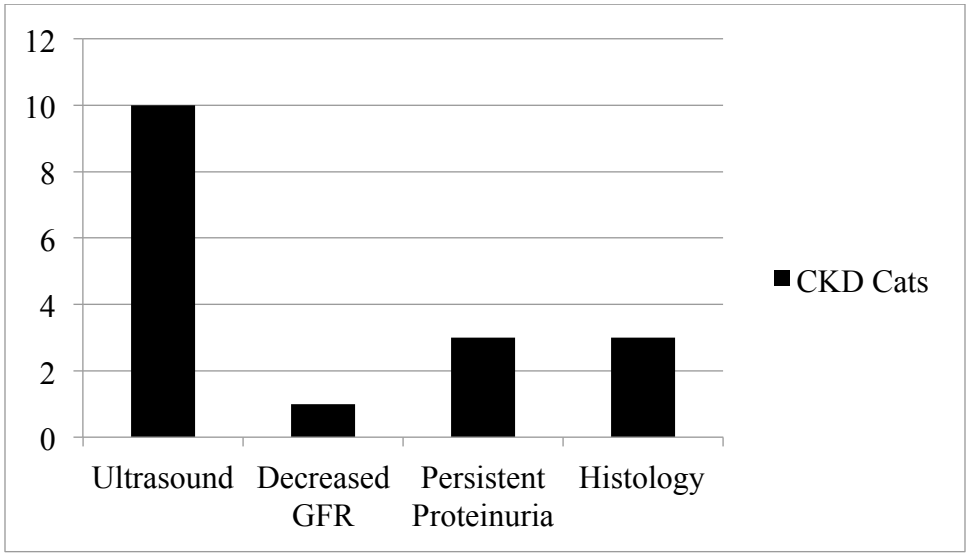
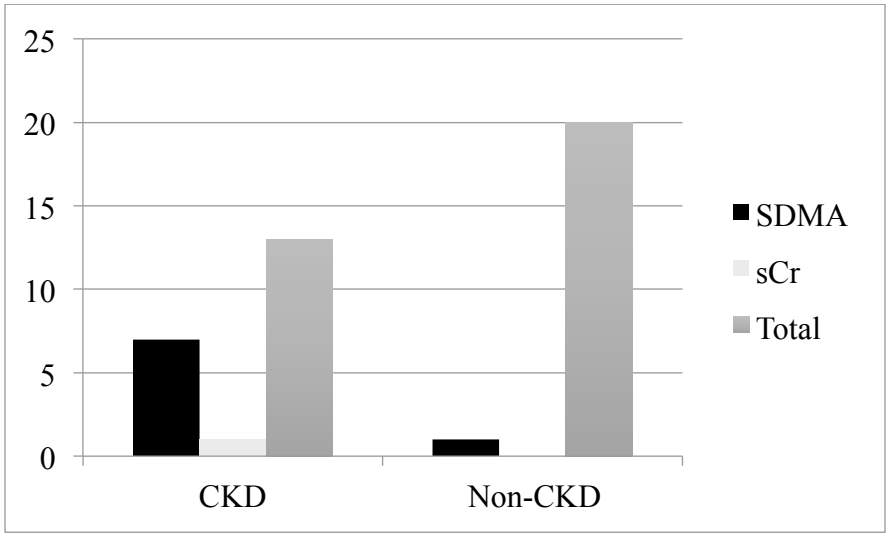


Figure 3.4 sCR versus SDMA increases in CKD and non-CKD cats



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Abbreviations

ACEi	Angiotensin-converting enzyme inhibitor
ADMA	Asymmetric dimethylarginine
AKI	Acute kidney injury
CHF	Chronic heart failure
CKD	Chronic kidney disease
CBC	Complete blood count
EDTA	Ethylenediaminetetraacetic acid
FIV	Feline immunodeficiency virus
GFR	Glomerular filtration rate
HPLC	High performance liquid chromatography
IL-6	Interleukin-6
IRIS	International Renal Interest Society
IV	Intravenous
Kg	kilogram(s)
KSU-VHC	Kansas State University-Veterinary Health Center
LC-MS	Liquid chromatography/mass spectrometry
MCT	Medium-chain triglycerides
MMA	monomethylarginine
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory
PCR	Polymerase chain reaction
PCV	Packed cell volume
SCr	Serum creatinine
SDMA	Serum symmetrical dimethyl arginine
SNGFR	Single nephron GFR
TNF-α	Tumor necrosis factor- α
TS	Total solids
TOD	Target organ damage
TP	Total protein

UPC	Urine protein to creatinine ratio
USG	Urine specific gravity
XLHN	X-linked hereditary nephropathy

Footnotes

[a] Advia 2120i Hematology system, Seimens Healthcare, Erlangen, Germany

[b] Cobas 6000 Hitachi c501, Roche Diagnostics, Indianapolis, IN

[c] Clinitek status analyzer, Seimens Healthcare, Erlangen, Germany

[d] Idexx Laboratories, Westbrook, ME

[e] Kansas State University Veterinary Diagnostic Laboratory

[f] Microsoft® Excel® for Mac 2011, Version 14.4.9 ©2008

[g] Ultrasonic Doppler Flow Detector, parks Medical Electronics Inc., Aloha, OR

[h] Michigan State University Diagnostic Center for Population and Animal Health, Lansing, MI

[i] TexaSoft, WINKS SDA Software, 7th Edition, Cedar Hill, Texas, 2011