Prediction of Ionized Calcium from Total Serum Calcium in Dogs

and

Exploration of the Role of Vitamin D Metabolites in Canine Calcium Oxalate Urolithiasis

A THESIS

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Elizabeth Marie Groth

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Eva Furrow (Advisor), Jody P. Lulich (Co-Advisor), and Dennis J. Chew

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Dedication

To my husband, Greg- You have been my rock and my never-failing support through this journey from college student to boarded internist. You have moved across the country (twice) without complaint and are still supportive and encouraging 10 years later. I could not have completed veterinary school, my internships, residency, and now my Master's without you by my side.

To my family- I love you all and your support has kept me going when I didn't think I could accomplish all I had dreamed of.

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Chapter 1: Introduction

Chapter one serves as an introduction to the complex metabolic pathways that regulate both calcium and vitamin D handling in the body. A portion of this chapter will be dedicated to the methodology of measuring of calcium and vitamin D metabolites. This chapter also serves as an introduction to calcium oxalate urolithiasis as an important medical condition in humans and animals that is often characterized by dysfunctional calcium handling. Chapters two and three are two separates studies performed at the University of Minnesota. Chapter two details a retrospective medical record review that further characterizes prediction of ionized hypercalcemia using total serum calcium. Chapter three details a prospective study evaluating the connection between canine calcium oxalate urolithiasis and alterations in vitamin D metabolism.

Calcium Metabolism: Biologic Roles and Regulation

Calcium is a critical mineral for a wide host of normal cellular functions. Calcium is required to perform enzymatic reactions, forming junctions between cells, as well as the conduction of electrical impulses in skeletal, cardiac, and smooth muscles. Calcium also plays a critical role in nerve transmission, bone formation, and cell growth and division. Without calcium, the body would not be able to function.

There are four major organs involved in the regulation of serum calcium: the parathyroid gland, the gastrointestinal tract, the kidneys, and bone.[1] There are complex feedback loops that connects these organs and a critical role is played by vitamin D and its metabolites, which will be detailed later in the chapter. Serum ionized calcium

concentration is the main driver in calcium regulation. Calcium is found in three different forms within the blood stream, but ionized is the biologically active form. The other forms include calcium bound to albumin and calcium complexed to anions such as phosphorus, sulfate, and citrate.

Because of the importance of ionized calcium in numerous physiologic roles, its concentration is maintained in a narrow range in healthy animals. The parathyroid gland and its primary hormone, parathyroid hormone (PTH), play the largest role in calcium regulation. Calcium sensing receptors on cell membranes detect high or low blood calcium concentrations and adjust PTH synthesis accordingly.[2] The main role of parathyroid hormone is to increase blood calcium concentrations by increasing activation of vitamin D to its most potent biologically important metabolite, calcitriol ($1,25(OH)_2D$). This stimulates absorption of calcium from the duodenum and upper jejunum and increases reabsorption of filtered calcium at the distal tubule. PTH also stimulates osteoclastic activity, which liberates calcium from the skeleton, increases resorption of calcium from the loop of Henle, and decreases reabsorption of phosphorus from the proximal tubule in the kidneys.[3]

PTH synthesis and release is inhibited by elevated ionized calcium as sensed by the calcium sensing receptors. Calcitonin is an important hormone in counteracting the actions of PTH, although there is no direct effect of calcitonin on PTH release. Calcitonin is produced by C-cells in the thyroid gland and acts primarily on osteoclasts to reduce liberation of calcium from the skeleton.[4] Calcitonin also has a small effect on the kidneys to reduce calcium reabsorption from the renal tubules. There is little to no effect of calcitonin on the regulated gastrointestinal absorption of calcium.

Calcium in Disease

Hyper- or hypo-calcemia can develop under a variety of pathologic states. Hypercalcemia in canines is often mediated by over production of PTH or secondary to a malignant process.[5,6] Tumors (benign or malignant) of the parathyroid gland can cause over production of PTH which increases the serum calcium concentrations above the level where renal excretion mechanisms can compensate. Anal gland tumors as well as lymphoma and mammary carcinomas are well documented to produce a peptide called parathyroid related peptide (PTHrP) whose actions mimic endogenously produced PTH.[7] Other tumor types have been reported to produce hypercalcemia, but the exact mechanism has not been clearly defined.

Other causes of hypercalcemia, which are less common, include hypoadrenocorticisim[8], vitamin D and metabolite toxicity, granulomatous disease[9], and renal dysfunction[10]. Renal dysfunction has been commonly documented to result in total hypercalcemia in dogs, but ionized hypercalcemia is rare. In fact, ionized hypocalcemia is more characteristic of kidney disease, particularly in late stages.[11,12] Late stage kidney disease in dogs can cause renal secondary hyperparathyroidism characterized by overstimulation of the parathyroid glands from chronic low-level hypocalcemia and lack of $1,25(OH)_2D$ due to reduced renal tissue and hyperphosphatemia. Secondary hyperparathyroidism is a compensatory mechanism for chronic hypocalcemia and rarely causes ionized hypercalcemia from overcompensation. Hypercalcemia of unknown etiology is the most common cause of hypercalcemia in the feline population; an idiopathic syndrome of hypercalcemia has not been reported in dogs.

Hypocalcemia is more common and of more varied in etiology than hypercalcemia in dogs. [13] Some clinically relevant causes include chronic kidney disease,[11] hypoparathyroidism[14], intestinal disease,[15] severe pancreatitis,[16] and as a result of non-specific critical illness.[17]

Measuring Calcium

The measurement of calcium can prove a diagnostic challenge due to the different types of calcium in circulation. The most commonly measured form of calcium is serum total calcium concentration. One of the current methodologies for total calcium measurement is spectroscopy following interaction with calcium-sensitive dye Arsenazo III.[18] Total calcium measurement is widely available on biochemical analyzers throughout the veterinary field and is therefore a common first-line screening tool. However, this measurement alone has been showed to be a poor predictor of ionized calcium in the past.[19] There have been several attempts to create formulas to correct total calcium and improve prediction of ionized calcium.[20,21] However, the older formulas are not currently recommended, and a more recent formula is cumbersome to calculate and has yet to be validated in the field.[21]

Measurement of ionized calcium is the preferred method to determine the biologically important fraction of circulating calcium. The equipment to measure ionized calcium using bench top analyzers is not as readily available as those to perform standard biochemical profiles. However, handheld point of care analyzers are becoming more prevalent in private veterinary practices. These hand-held analyzers are used to measure electrolytes and/or blood gases and frequently are capable of measuring ionized calcium.

Ionized calcium is measured with ion-selective electrode potentiometry. This methodology has not yet to be implemented in automatized routine serum chemistry analyzers. It would be clinically useful if ionized calcium could be accurately predicted from total serum calcium which is more frequently measured.

Vitamin D Metabolism

Vitamin D and its metabolites play a critical role along with PTH in maintaining circulating ionized calcium concentrations within a narrow range in health. As reviewed above, some of the effects of PTH on target tissues are mediated through vitamin D activation to $1,25(OH)_2D$. Circulating vitamin D exists in two forms: D_2 (ergocalciferol) and D_3 (cholecalciferol). Vitamin D_2 accounts for only a small percentage of vitamin D in the circulation of animals eating mostly meat. Vitamin D_2 undergoes the same metabolism as vitamin D_3 and may be less bioactive, although this is controversial and limited data is available in veterinary speces.[22] The two circulating forms are reported as a total 25(OH) vitamin D which is a hydroxylated metabolite of vitamin D. In the dog and cat, almost all of the total 25(OH)D exists from $25(OH)D_3$ due to the large proportion of D_3 in the diet. Vitamin D undergoes several steps to convert it from the inactive form to the biologically active $1,25(OH)_2D$ metabolite (Figure 1).

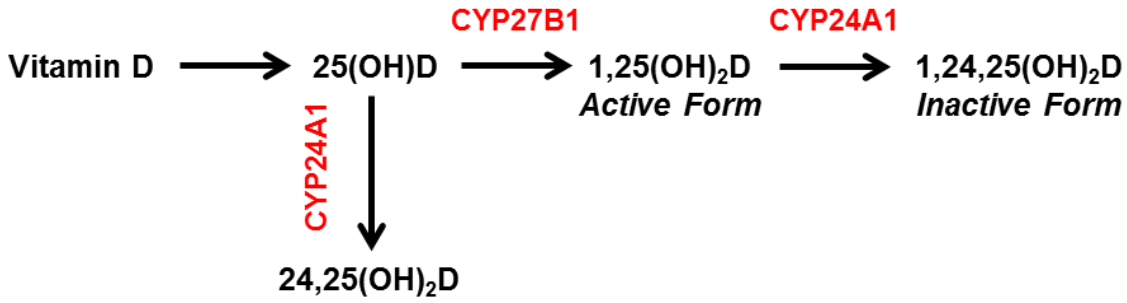

Figure 1: Vitamin D metabolism from dietary intake through activation and inactivation.

In humans, the major source of vitamin D is conversion of pro-vitamin D to vitamin D through an enzyme present in the skin that is activated by UV radiation and heat. This pathway is not present in dogs and cats, and these species are therefore reliant on dietary intake for vitamin D. Vitamin D is absorbed into systemic circulation through the GI tract where is carried to the liver bound to vitamin D binding protein (VDBP). In the liver, vitamin D is hydroxylated via 25-hydroxylase to 25(OH)D. From the liver, 25(OH)D re-enters circulation as a still relatively inactive metabolite bound to VDBP. The final step in converting partially active 25(OH)D to active vitamin D requires the enzyme 1 alpha hydroxylase (CYP27B1) predominately present in renal tissue. This enzyme converts 25(OH)D to the most active metabolite $1,25(OH)_2D$ which then enters circulation. The expression of this enzyme is related to a number of hormones including PTH, fibroblast growth factor-23, calcitonin and is affected by circulating calcium and phophorus.[23,24]

The primary role of $1,25(OH)_2D$ is to increase calcium and phosphorus absorption from the GI tract. This absorption is mediated by transcellular transport of calcium from the GI lumen into circulation via calcium binding proteins (calbindin and calcium transport proteins). Synthesis and expression of these proteins are regulated via binding

of $1,25(OH)_2D$ to vitamin D receptors (VDR) present on the enterocytes. During periods of high calcium intake, less 25(OH)D is activated into $1,25(OH)_2D$ and therefore expression of both calbindin and calcium transport proteins are downregulated. The reverse is also true. In times of low calcium intake, this system is upregulated. Vitamin D independent mechanism of calcium absorption also exist. [25,26]

When circulating $1,25(OH)_2D$ exceeds the body's needs or there is excess 25(OH)D in circulation, both can be converted to metabolites that are less biologically active. The primary enzyme responsible for deactivating vitamin D is 24 hydroxylase (CYP24A1) (Figure 1). This enzyme converts $1,25(OH)_2D$ to $1,24,25(OH)_3D$ and 25(OH)D to $24,25(OH)_2D$. Regulation of 24 hydroxylase is primarily mediated by FGF-23. Both deactivated metabolites are then excreted in the urine bile.

In addition to the primary role played in calcium metabolism, vitamin D and its metabolites also have several important functions elsewhere in the body. Vitamin D receptors are present in a variety of tissues other than the GI tract and renal tissue. VDR expression is important in the parathyroid glands to maintain genomic control of PTH synthesis. Cardiac muscle, immune cells, pancreatic beta cells, and osteoblasts also express VDR as well as many other tissues. [27–30] Vitamin D metabolite interaction with VDR is critical to the health of these tissues and when abnormalities in VDR occurs, clinically significant abnormalities in calcium and vitamin D metabolism can develop. Some of these will be discussed in more detail in the next section.

Vitamin D in Disease

Because of the critical role vitamin D plays in intestinal calcium absorption, there are significant consequences of gastrointestinal disease on vitamin D homeostasis. Hypocalcemia is often reported in patients with protein losing enteropathies of various etiology. In part, this is due to the decrease in the fraction of calcium bound to albumin (total hypocalcemia) as well as loss of calcium into the GI tract with albumin. However, protein-losing enteropathies are also associated with a decreased in ionized calcium, suggesting that there are other mechanisms that contribute to hypocalcemia. Proposed mechanisms include a decrease in voluntary intake or malabsorption of calcium and/or vitamin D3. Recent studies in dogs have shown lower 25(OH)D concentrations in affected dogs as well as poorer outcomes associated with lower 25(OH)D concentrations.[15,31,32] There is also some evidence in people that vitamin D is required to maintain tight junctions between enterocytes and deficiency can increase permeability of the gut, leading to progressive disease.[33] However, this finding has not been shown yet in dogs.[34]

The kidney is the major site of 1 alpha hydroxylase activity. As such there are significant changes in vitamin D and calcium metabolism that occur during the course of renal parenchymal loss in chronic kidney disease. There have been numerous studies in humans as well as recent literature in dogs evaluating the link between vitamin D metabolites and chronic, progressive renal disease. In dogs, as renal disease progresses, vitamin D concentrations decrease.[12] This includes both $1,25(OH)_2D$ and 25(OH)D. This is likely a multifactorial phenomenon including decrease intake of 25(OH)D and decreased conversion of 25(OH)D to $1,25(OH)_2D$ due to decreased 1 alpha hydroxylase activity. Hyperphosphatemia plays a major role in this process through direct inhibitory

effects on 1 alpha hydroxylase and by increasing FGF-23. Increased complexed form of calcium with phosphorus and citrate also contribute. Together these processes cause an ionized hypocalcemia that is commonly seen in late stage renal disease in dogs.[35]

Vitamin D receptors are expressed in cardiac muscle suggesting important role of VDR in cardiac health. Decreased circulating 25(OH)D has been shown to occur in late stage degenerative mitral valve disease in dogs as well as dogs in congestive heart failure.[36] Vitamin D deficiency, as determined by total 25(OH)D, is also commonly reported in human heart disease with a strong association between deficiency and degenerative valve disease, although a cause and effect relationship has not been established.[37]

Hypervitaminosis D is uncommon in animals and typically occurs as a result from excessive dietary supplementation, ingestion of human medications that contain calcitriol or calcitriol analogs, or ingestion of toxins, such as rodenticide, or medications that contain cholecalciferol.[38] Vitamin D toxicity, depending on the formulation (i.e. 25(OH)D vs. $1,25(OH)_2D$), can result in hypercalcemia and commonly hyperphosphatemia. This increase in circulating calcium and phosphorus can develop rapidly to life threatening renal failure due to soft tissue mineralization if not treated appropriately.

Measuring Vitamin D Metabolites

There are several methodologies by which vitamin D and its metabolites can be measured. These include radioimmunoassay, liquid chromatography/mass spectrometry, and chemiluminescence. Radioimmunoassay (RIA) is available for both 25(OH) and

$1,25(OH)_2D$ and uses and ^{125}I labeled antibody to the desired metabolite. These assays do not differentiate between vitamin D_3 and vitamin D_2 metabolites but are accurate as compared to the gold standard of measurement using liquid chromatography. Liquid chromatography with concurrent mass spectrometry is available for most metabolites including 25(OH)D, $1,25(OH)_2D$ and $24,25(OH)_2D$ and uses direct detection methods. Chemiluminescence uses a isoluminol tracer that binds to vitamin D and is available for 25(OH)D and $1,25(OH)_2D$.

Chemiluminescence is the preferred methodology in people and is FDA approved. Initial data for its use in dogs has shown acceptable performance, but this data is limited.[39,40] Currently, liquid chromatography with concurrent mass spectrometry is considered a gold standard assay in humans for measuring vitamin D metabolites.[41] When sample volume is limited, such as with pediatrics or small patients, RIA also has acceptable performance. Because this method uses radioactive isotopes, there is concern for lab personnel and therefore test kits using this method are becoming less widely available. There is currently no data directly comparing the various methods in animals used to measure vitamin D metabolites. The vitamin D external quality assessment scheme (DEQAS) serves as a monitoring board to ensure quality and accuracy of vitamin D metabolite measurements, but no veterinary lab has yet to achieve this cirtification.[41]

Calcium Oxalate Urolithiasis

Calcium oxalate (CaOx) urolithiasis is a problem that has both human and animal health implications. It is estimated that approximately 80% of human nephroliths are composed of CaOx, although composition is not always determined in human medicine.[42]

In dogs, just under half of the stones submitted to the University of Minnesota Urolith Center were CaOx as of 2013 and there has been an overall increasing prevalence over the last decade. [43] In animals, CaOx stones cause a wide range of issues varying in severity from mild cystitis to life threatening urinary obstruction. The causes of CaOx urolithiasis is multifactorial but the underlying pathology most often relates to excess calcium excretion (hypercalciuria). Increased urinary oxalate excretion (hyperoxaluria) is reported in humans as a contributing cause of CaOx stone formation.[44] In dogs, primary hyperoxaluria has been reported as a genetic disease in Coton de Tuler dogs.[45] However, hyperoxaluria is uncommon in the wider population of stone forming dogs.[46,47]

Hypercalciuria is typically defined as an increased urinary calcium excretion in milligrams per kilogram over a 24-hour period. However, in veterinary medicine, hypercalciuria is typically defined as an increased calcium to creatinine ratio (UCaCr) on a single urine sample. Hypercalciuria is the major driver behind human CaOx stones and is also a common metabolic disturbance in CaOx stone-forming dogs.[47]

Hypercalcemia is one cause of hypercalciuria. It occurs in response to an increased filtered load of calcium into tubular fluid. However, most human and canine CaOx stone formers have normal blood calcium concentrations. Hypercalciuria is termed idiopathic in this scenario. There are three main mechanisms by which idiopathic hypercalciuria can occur: decreased renal resorption of calcium (e.g. “renal leak”), increased bone resorption with compensatory renal excretion, and increased intestinal absorption with compensatory renal excretion. The exact mechanism by which hypercalciuria occurs in dogs is currently unknown. Other previously identified risk factors for CaOx stone formation include middle to older age, male sex, breed, and neutered status.[48]

Renal leak hypercalciuria is a result of abnormally decreased resorption of calcium in the renal tubules, although the precise location within the tubules may vary with disease type. This decreased tubular resorption is the cause of CaOx urolithiasis in about 5-10% of human cases and is diagnosed based on the presence of hypercalciuria in conjunction with low to low-normal serum calcium and high-normal to high PTH concentration.[49] It can occur secondary to identifiable causes such as malformation of the kidney (e.g. medullary sponge disease) or genetic defects in ion channels such as X-linked recessive nephrolithiasis (e.g. Dent disease).[50,51] However, there are many cases where an underlying cause is not identified. This mechanism of disease has not yet been identified in dogs. Many dogs with CaOx urolithiasis have high-normal blood ionized calcium, which is not consistent with a renal leak disorder.[47]

Increased bone resorption can also cause hypercalciuria. The rate of bone resorption is primarily mediated by PTH, but PTH-independent pathways can also increase the rate of resorption as well. As resorption of calcium from the skeleton increases, more calcium and phosphorus are released into the bloodstream and the kidneys compensate by decreasing absorption and increasing excretion of the excess minerals into the urine. Increased skeletal calcium resorption has been identified in humans as a risk factor for CaOx stone formation and is associated with decreased bone mineral density.[52,53] Minimal data is available in canines, but unpublished data from our lab showed decreased bone resorption in dogs with CaOx urolithiasis.

The last mechanism of idiopathic hypercalciuria is increased intestinal absorption of calcium. This increased absorption is coupled with compensatory renal excretion similar to increased bone resorption. Regulated gastrointestinal absorption of calcium is

mediated via vitamin D and its metabolites as described in a previous section. Recently, alterations in vitamin D metabolism has been showed to increase risk of CaOx nephrolithiasis.[54] Specifically, patients with nephroliths had higher ratios of 25(OH)D to 24,25$(OH)_2$D than non-stone formers. This indicates an inability to appropriately convert active to inactive vitamin D metabolites. Recessive mutations of *CYP24A1*, the gene that encodes 24 hydroxylase, have been identified as the underlying cause of this alteration in some patients, but mutations in this gene do not explain the subtler deficiency present in most stone formers.[55]

It has been proposed that increased expression of VDR in cells increases tissue response to normal levels of 1,25$(OH)_2$D.[29] Normocalcemic hyperparathyroidism is another documented cause of increased intestinal absorption. [56] PTH is not routinely measured in patients without hypercalcemia, and this condition may be underdiagnosed. However, it is unlikely to be a major cause of hypercalciuria in dogs, as PTH concentrations have been reported to be normal in most dogs with hypercalciuria.[46]

Hypotheses/Aims

Hypothesis 1 (Chapter 2) – There is an optimal threshold of total calcium above which is highly predictive of ionized hypercalcemia in dogs and this threshold will be higher when azotemia is present.

Specific Aim 1 – Determine the accuracy of tCa for predicting hypercalcemia across multiple cut-off thresholds in dogs with and without azotemia.

Hypothesis 2 (Chapter 3) – Dogs with a history of CaOx urolithiasis have higher concentrations of $1,25(OH)_2D$ and higher ratios of $25(OH)D/24,25(OH)_2D$ compared to control dogs without urolithiasis.

Specific aim 2 – Evaluate vitamin D metabolites ($25(OH)D$, $1,25(OH)_2D$, and $24,25(OH)_2D$) in 20 dogs with a history of CaOx urolithiasis and in 20 age-, sex-, and breed-matched stone-free control dogs.

Chapter 2: Determination of a serum total calcium threshold for the accurate prediction of ionized hypercalcemia in dogs with and without azotemia

Background: Total serum calcium concentrations (tCa) are reported to be poorly predictive of ionized calcium (iCa) status in dogs.

Hypothesis: There is an optimal threshold of tCa above which is highly predictive of ionized hypercalcemia and this threshold will be higher in azotemic dogs as compared to non-azotemic dogs.

Animals: Non-azotemic (n=1,381) and azotemic (n=542) dogs presenting to a university teaching hospital.

Methods: Retrospective medical record review of all dogs presenting for evaluation over a 5-year period with paired tCa and iCa measurements. Positive and negative predictive values, sensitivity, and specificity were calculated for tCa cut offs above 11.0 mg/dL in both non-azotemic and azotemic groups.

Results: In the non-azotemic group, an optimal tCa threshold of 12.0 mg/dL resulted in a positive predictive value of 95% (95% CI: 83-99%) for ionized hypercalcemia. The optimal tCa threshold was greater in the azotemic group at 14.0 mg/dL, resulting in a positive predictive value of 94% (95% CI: 70-100%). The prevalence of ionized hypercalcemia did not differ between the groups (<10% in both, $P = .31$), but ionized hypocalcemia was more prevalent in the azotemic group than the non-azotemic group (45% versus 21%, respectively; $P < .001$).

Conclusions and Clinical Importance: Total calcium concentrations at or above the optimal thresholds identified in this study are strongly predictive of ionized hypercalcemia and should prompt further diagnostics to determine the underlying cause

of hypercalcemia. Ionized calcium concentrations should be evaluated on patients with tCa below the thresholds if there is clinical suspicion for calcium abnormalities.

Introduction

Hypercalcemia in dogs is often an indicator of serious disease with the most common cause being malignant neoplasia such as lymphosarcoma or anal gland adenocarcinoma.[6] Less common causes of hypercalcemia include hypoadrenocorticism, primary hyperparathyroidism, and hypervitaminosis D.[57] Persistent hypercalcemia, regardless of underlying cause, can have serious consequences including soft tissue mineralization and subsequent renal failure. The serious nature of such diseases and complications requires an accurate way to detect hypercalcemia so that a diagnosis can be made and appropriate intervention can be instituted as soon as possible. Ionized calcium (iCa) is the biologically active form and is the most relevant to clinical practice.[57] However, iCa measurements are not readily available in all practice settings, and serum total calcium (tCa) is often used in its place which is of questionable acceptability.

A large study in dogs showed significant diagnostic discordance between tCa or adjusted/corrected calcium and iCa status. This study evaluated a single tCa threshold for defining hypercalcemia (12 mg/dL), based on the upper limit of the laboratory reference interval.[13] The positive predictive value (PPV) at this threshold was relatively low at 68%. Dogs with ionized hypercalcemia can have tCa that are normal to maximally elevated, but median tCa have been reported to be well above the above reference intervals.[58] This suggests that there may be an optimal cut off for tCa that can more reliably predict hypercalcemia in dogs.

When exploring the optimal diagnostic threshold for tCa, its utility for predicting ionized hypercalcemia should be examined separately in subpopulations of patients with and without azotemia. Previous reports have shown that tCa was less predictive in dogs

with chronic kidney disease as compared to dogs that were non-azotemic.[10] In fact, in dogs with kidney disease, using the upper limit of the laboratory reference interval as the tCa threshold resulted in a PPV of only 27% for predicting ionized hypercalcemia. Azotemia affects the diagnostic discordance between tCa and iCa due to increased complexed calcium.[35] Therefore, it is important to reinvestigate the use of tCa in populations categorized as non-azotemic or azotemic based on standard biomarkers that can be easily applied in a clinical setting.

The objective of this study was to evaluate the accuracy of tCa for predicting ionized hypercalcemia across multiple tCa thresholds in dogs with and without azotemia. Our hypothesis was that there is a threshold for tCa which is strongly predictive of ionized hypercalcemia. We predicted that this threshold would be higher in patients with azotemia compared to those without.

Materials and Methods

Case Selection

Medical records were reviewed from the University of Minnesota Veterinary Medical Center (UMN VMC) between 2011-2016 for patients with measurements of both of serum tCa and blood iCa collected within 12 hours of one another. Information obtained from the medical records included age, breed, sex, time between iCa and tCa sampling, BUN, and creatinine. All cases were divided into two groups based on the presence of azotemia. Azotemia was defined as either BUN >31 mg/dl or creatinine >1.6 mg/dl based on the upper limits of the laboratory reference intervals. Further characterization of azotemia was not performed. If multiple pairs of calcium

measurements were obtained on an individual dog during a single visit, only the pair with the shortest time between sample collection was included in the analysis. Samples from the same dog were included in analysis if the samples were obtained during different hospital visits (discharged in between). All iCa measurements were obtained using a hand-held analyzer (iSTAT 1, Abbott Point of Care Inc, East Windsor, NJ) with a canine reference interval of 5.1 - 5.9 mg/dL. Total serum calcium was measured using standard reference laboratory methods (AU480® Chemistry analyzer, Beckman Coulter, Inc., Brea, CA.); the canine reference interval was 9.3 - 11.5 mg/dL.

Statistical Analysis

Thresholds of tCa were selected in 0.5 mg/dL increments between 11.0 and 15.0 mg/dL. Sensitivity, specificity, PPV and negative predictive value (NPV) were calculated at each of these thresholds for both the non-azotemic and azotemic groups. To determine an optimal tCa threshold for predicting hypercalcemia, the PPVs were evaluated to identify the lowest threshold with a PPV >90%; 95% confidence intervals (Clopper-Pearson exact method) for PPVs were calculated. Comparison of proportions of ionized hyper-, hypo-, and normocalcemia between the non-azotemic and azotemic groups were performed using a Cochran Armitage trend test, followed by Chi-squared tests to evaluate each status individually. A Chi-squared test was also used to compare the proportion of pediatric (<1-year-old) dogs between the between true positives and false negatives in the non-azotemic group. Student's *t*-tests were used to compare age, iCa, and tCa between non-azotemic and azotemic groups and to compare iCa between the true positives and

false negatives. All calculations were carried out using R software for statistical computing (R, version 3.3.1. www.r-project.org).

Results

A total of 2,334 medical records were reviewed. Four hundred and eleven visits were excluded from analysis: 404 were excluded due to multiple samples for an individual dog within the same hospital visit, and 7 were excluded due to incomplete medical records. The remaining 1,923 visits from 1,765 dogs were included for analysis. The non-azotemic group comprised 1,381 visits and the azotemic group comprised 542 visits. A total of 124 breeds were represented. The median time between the tCa and iCa measurements was 43 minutes (range 0 minutes to 12 hours).

Data including age, calcium concentrations, and prevalence of ionized hyper-, hypo-, and normocalcemia for the non-azotemic and azotemic groups are presented in Table 1. The non-azotemic group was younger than the azotemic group (P <.001). The prevalence of ionized hypocalcemia was significantly higher in the azotemic group than the non-azotemic group (P <.001). There was no difference in prevalence of ionized hypercalcemia between the groups (P = .31).

Table 1: Patient population characteristics including calcium concentrations and disease prevalence based on iCa.

Parameter	**Non-Azotemic** (n=1,381)	**Azotemic** (n= 542)	**P Value**
Age (Range), years	7.7 (.1-15.9)	9.3 (0.1-18.5)	**<.001**
tCa Mean (Range), mg/dL	9.7 (3.3-17.1)	9.8 (4.4-19.3)	**.019**
iCa Mean (Range), mg/dL	5.3 (2.3-9.5)	5.0 (1.8-9.7)	**<.001**
Ionized calcium status			**<.001**
Hypercalcemic	6.6% (n=92)	8.1% (n=44)	.31
Hypocalcemic	20.7% (n=286)	45.2% (n=245)	**<.001**

P values in bold denote significance (<0.05).

The PPVs increased rapidly for tCa thresholds above 10 mg/dL. The rate of increase was greater in the non-azotemic group as compared to the azotemic group and plateaued sooner (Figure 1). For the non-azotemic group, the optimal tCa threshold for predicting ionized hypercalcemia was 12.0 mg/dl with a PPV of 95% (95% CI: 83 – 100%). The NPV for this threshold was 96%, and the sensitivity and specificity were 41% and 100% respectively. For the azotemic group, the optimal tCa threshold for predicting hypercalcemia was 14.0 mg/dL with a PPV of 94% (95% CI: 70-100%). The NPV at this threshold was 94%, and the sensitivity and specificity of this threshold was 34% and 100% respectively. Figure 2 shows data from individual dogs in relation to tCa, iCa, and the optimal thresholds. The sensitivity, specificity, PPV and NPV at each of the evaluated thresholds are presented in Tables 2 (non-azotemic group) and 3 (azotemic group).

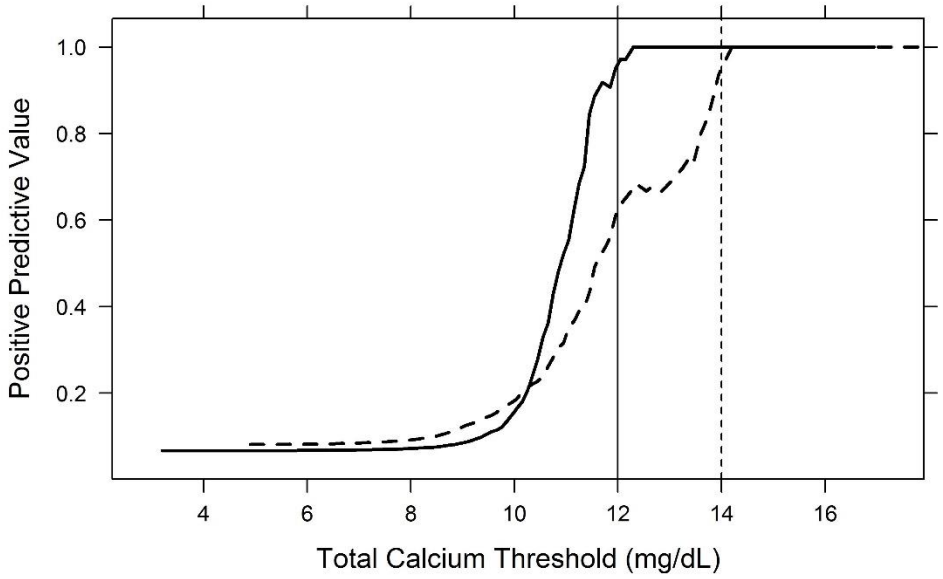

Figure 2: PPV of both non-azotemic dogs (solid black line) and azotemic dogs (dotted black line) over the range of evaluated threshold values. The vertical dotted lines represent the designated thresholds for non-azotemic dogs (12 mg/dL, solid black) and for azotemic dogs (14 mg/dL, dotted black)

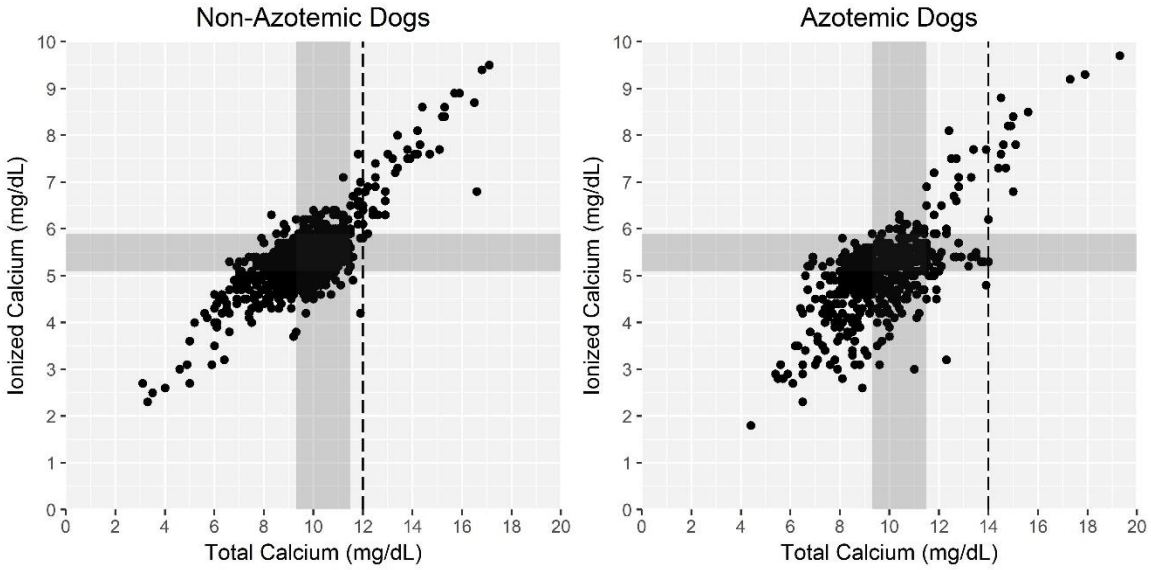

Figure 3: Scatter plot representation of non-azotemic and azotemic dogs. Each dot represents an individual dog. The shaded area on each plot represents the values within the reference intervals of ionized and total calcium. The dotted line proposed thresholds for both groups.

Table 2: PPV, NPV, sensitivity, and specificity data for all thresholds for the non-azotemic group. The optimal threshold is represented in bold.

Threshold	PPV (95% CI)	NPV	Specificity	Sensitivity
11	.52 (.43-.62)	.97	.96	.64
11.5	.84 (.73-.93)	.97	.99	.53
12	**.95 (.83-0.99)**	**.96**	**1.00**	**.41**
12.5	1.00 (.89-1.00)	.95	1.00	.34
13	1.00 (.86-1.00)	.95	1.00	.26
13.5	1.00 (.82-1.00)	.95	1.00	.21
14	1.00 (.79-1.00)	.94	1.00	.17
14.5	1.00 (.72-1.00)	.94	1.00	.12
15	1.00 (.69-1.00)	.94	1.00	.11

Table 3: PPV, NPV, sensitivity, and specificity data for all thresholds for the azotemic group. The optimal threshold is represented in bold.

Threshold	PPV (95% CI)	NPV	Specificity	Sensitivity
11	.32 (.23-.41)	.98	.84	.84
11.5	.43 (.32-.55)	.97	.92	.73
12	.61 (.45-.75)	.97	.96	.64
12.5	.68 (.50-.82)	.96	.98	.57
13	.69 (.48-.86)	.95	.98	.41
13.5	.73 (.49-.89)	.95	.99	.36
14	**.94 (.70-1.00)**	**.94**	**1.00**	**.34**
14.5	1.00 (.75-1.00)	.94	1.00	.30
15	1.00 (.59-1.00)	.93	1.00	.16

We further evaluated the population of dogs with false negatives in the non-azotemic group by comparing them to the non-azotemic true positives. Twenty-nine percent (13/45) of the false negatives were in pediatric dogs compared with 2% (1/47) of the true positives ($P < .001$). Thus, false negatives were highly prevalent in pediatric dogs; 93% (13/14) of those with ionized hypercalcemia were incorrectly classified based on the tCa compared with 41% (32/78) of adult dogs (OR = 19, 95% CI 2 - 150, $P < .001$). Dogs with false negatives also had a significantly lower iCa (mean = 6.2 ± 0.2 mg/dL) than those with true positives (mean = 7.4 ± 0.9 mg/dL, $P < 0.001$) in both groups.

Discussion

The data from this study demonstrate that tCa thresholds of 12.0 mg/dL in non-azotemic and 14.0 mg/dL in azotemic dogs are highly predictive of ionized hypercalcemia. Due to the low prevalence of ionized hypercalcemia, the NPV was high at all thresholds. However, the sensitivity (ability to correctly classify patients with disease as positive) of tCa for detecting ionized hypercalcemia was low. Thus, if there is clinical suspicion for hypercalcemia, an iCa should be evaluated in the face of a normal tCa.

There was a clinically relevant difference in optimal threshold values in non-azotemic versus azotemic patients. Azotemia can affect calcium metabolism in a variety of ways. Azotemia secondary to renal dysfunction is associated with increased phosphorus, which is one of the common anions that complexes with calcium. This can increase the tCa but has the opposite effect on iCa. Renal dysfunction can also result in renal secondary hyperparathyroidism, but ionized hypocalcemia is more common than

ionized hypercalcemia due to reduced calcitriol synthesis and the aforementioned effect of hyperphosphatemia on iCa.[11,13] Studies have shown that approximately one third of dogs with chronic kidney disease have ionized hypocalcemia whereas <10% have ionized hypercalcemia.[11,13] The data in our study are consistent with these studies. The prevalence of ionized hypercalcemia was low in azotemic dogs (8%), but the prevalence of ionized hypocalcemia was high (45%). Of note, tCa tends to overestimate iCa in chronic kidney disease, resulting in false negative calls for ionized hypocalcemia and false positive calls for ionized hypercalcemia. Thus, tCa above the reference interval has been shown to be poorly predictive of ionized hypercalcemia in dogs with chronic kidney disease with a PPV of only 27%.[13]

A recent study in cats with and without azotemic chronic kidney disease showed similar performance of tCa in predicting ionized hypercalcemia.[59] This study found a moderate correlation between tCa and iCa in both non-azotemic and azotemic cats (r = 0.6 for both). They also found that tCa above the reference range (11.8 mg/dL) was very specific for ionized hypercalcemia in both non-azotemic and azotemic feline patients (100% and 99.9% respectively), but the sensitivity was low at 9% in the non-azotemic group and 28% in the azotemic group. If the tCa threshold was lowered to 10.5 mg/dL, which was below the laboratory reference range, it remained highly specific in the non-azotemic group (90%) with a greatly improved sensitivity (95%). A lower tCa threshold of 10.3 mg/dL also improved sensitivity in the azotemic group (72%) but reduced the specificity (71%). However, while these reduced thresholds optimize the combination of sensitivity and specificity, they do not take into account the effects on the PPV. The PPV is important, as it estimates the proportion of patients with a tCa above the threshold that

will have ionized hypercalcemia (proportion of positive tests that are true positives). The study did not directly report PPV, but sufficient data is provided to calculate it. The 10.5 mg/dL threshold in the non-azotemic group had a PPV of 56%, and the 10.3 mg/dL threshold in the azotemic group had a PPV of 31%. These tCa thresholds are therefore too low for accurate prediction of ionized hypercalcemia, as half or more of the tCa above the thresholds are false positives. The results also further confirm the poorer performance tCa for the prediction of iCa in azotemic patients.

The most important clinical implication from our study is the recognition that an increase in tCa above 12.0 mg/dL is often indicative of ionized hypercalcemia in a non-azotemic dog. Several neoplastic, as well as non-neoplastic, diseases can cause hypercalcemia and early recognition of these diseases could lead to earlier interventions and potentially increased length of life. A tCa above 12.0 mg/dL in a non-azotemic adult dog should not be ignored but rather should prompt further investigation including confirmation of ionized calcium status to see if it is concordant or not.

Another relevant finding from our study is that an increased threshold of > 14.0 mg/dL total calcium is associated with an increased ionized calcium in most azotemic dogs. Performance of tCa using the upper limit of the reference interval was a poor predictor of ionized hypercalcemia (PPV = 43% at a tCa threshold of 11.5 mg/dL); many azotemic dogs had mild tCa elevations without concurrent iCa elevations. By analyzing multiple thresholds, we were able to determine an optimal tCa value, 14 mg/dL, that has much better performance for prediction of ionized hypercalcemia in the azotemic population, although this came at the expense of a low sensitivity (34%).

Findings from our study may allow expanded analysis in retrospective research studies conducted in the future. The poor correlation between tCa and iCa has historically made it difficult to interpret calcium status in studies where only tCa was reported. Using the upper limit of the laboratory reference interval for tCa has a reasonably high PPV for non-azotemic dogs. In contrast, more than half of azotemic dogs with an elevated tCa do not have an ionized hypercalcemia. Failure to account for the poor performance of tCa in the context of azotemia could confound the ability to detect the impact of hypercalcemia on clinical questions of interest. For example, one study on mediastinal lymphoma found no association between the presence of hypercalcemia and survival.[60] However, more than half of the dogs classified as hypercalcemic had only tCa data available, and the authors state that azotemia was common (present in 48% of hypercalcemic dogs). Classifying hypercalcemia based on tCa is common practice in veterinary oncologic studies. Unlike the aforementioned mediastinal lymphoma study, other canine lymphoma studies, as well as anal gland tumor studies, have reported hypercalcemia to be a prognostic factor.[61,62] Without confirming ionized hypercalcemia, incorrect prognostic information may be conveyed to owners based on the reported tCa, particularly if the dog is azotemic. We propose that when iCa is not available (e.g. retrospective studies), the thresholds determined in this study could be useful as surrogates to classify calcium status separately for non-azotemic and azotemic populations.

A recent study developed a model for predicting iCa in dogs based on numerous values available on a biochemical profile in the dog.[21] This model had a high PPV (90%) and specificity (99.6%), and a moderate sensitivity (64%). This model provides results that are more sensitive than the optimal tCa threshold identified in the present study with

a comparable PPV, But the model can be cumbersome to enter variables into the equation used to generate an estimated ionized calcium. Use of this model has not been validated with variables measured from multiple laboratories, which would be critical prior to application in a clinical setting as models often perform worse on external validation.[63]

This study has several limitations because of its retrospective nature. While most tCa and iCa measurements were run in less than one hour of each other, we allowed for up to 12 hours between measurements. Therapies that patients received during this time period which may have altered one or both measurements were not recorded. We also did not determine whether azotemia was due to structural renal disease (such as chronic kidney disease) or not, nor did we consider the magnitude of azotemia. It is possible that our thresholds would have been altered if we further differentiated between renal and pre- or post-renal azotemia and the degree of azotemia that was present. An advantage of our simplified methods is that our threshold can be applied to interpretation of tCa in an azotemic patient, regardless of whether or not a urinalysis is available at the same time point to classify their azotemia. Finally, as discussed above, we selected tCa thresholds based on PPV rather than sensitivity and specificity. The PPV informs the clinical interpretation of a positive test result when the gold standard is not available, but unlike sensitivity, it varies based on disease prevalence. When a disease is common, the PPV will increase and, vice versa, a low prevalence will decrease the PPV. Therefore, the PPV for the thresholds designated in this study may vary in other patient populations. Many of the cases included in this study were hospitalized patients, and the prevalence of idiopathic hypercalcemia could be different in patients presenting for outpatient evaluation.

In conclusion, this study showed that serum tCa concentrations at or above the designated thresholds are highly predictive of ionized hypercalcemia in dogs with and without azotemia. This information has significant case management and research implications. Total calcium thresholds could be used as a surrogate marker of calcium status when iCa is not available. However, it is still recommended to perform an iCa in all dogs to confirm calcium abnormalities. Furthermore, although there are thresholds that are highly predictive of ionized hypercalcemia, a normal tCa should not be used to exclude ionized hypercalcemia in dogs. Additional studies are needed to further validate these thresholds using other reference laboratories and patient populations.

Chapter 3: Vitamin D Metabolism in dogs with and without calcium oxalate urolithiasis

Background: Abnormalities in vitamin D metabolism have been reported in people with calcium nephrolithiasis, but limited data is available on vitamin D status in dogs with calcium oxalate (CaOx) urolithiasis.

Objective: To compare vitamin D metabolites in dogs with and without CaOx urolithiasis.

Animals: Forty dogs with (n=20) and without (n=20) a history of CaOx urolithiasis and hypercalciuria.

Methods: Serum 25-hydroxyvitamin D [25(OH)D], 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], and 24,25-dihydroxyvitamin D [$24,25(OH)_2D$] were measured. The ratios of $25(OH)D/24,25(OH)_2D$ and $1,25(OH)_2D$ /25(OH)D were calculated and statistically compared between cases and controls.

Results: There were no significant differences between cases and controls when comparing 25(OH)D, $24,25(OH)_2D$, $1,25(OH)_2D$, or $1,25(OH)_2D/25(OH)D$. Cases had higher $25(OH)D/24,25(OH)_2D$ compared to controls (median = 1.43 and 1.17, respectively; p= .012). There was overlap in the ranges for $25(OH)D/24,25(OH)_2D$ between cases and controls, but 7 cases (35%) had ratios above the control dog range. There was a moderate positive correlation between the ratio of $25(OH)D/24,25(OH)_2D$ and urinary calcium-to-creatinine ratios (r = .37, p = .017).

Conclusions and Clinical Importance: This data suggests that decreased conversion of 25(OH)D to $24,25(OH)_2D$ occurs in a subset of dogs with CaOx urolithiasis. Abnormalities in vitamin D metabolism may contribute to stone risk in dogs.

Introduction

Calcium oxalate (CaOx) urolith formation has a multifactorial etiology that is not well understood. In both people and dogs, excess excretion of calcium into the urine is a significant risk factor, but the mechanism behind hypercalciuria is poorly understood. There are a number of factors including diet and genetics that likely influence the degree of hypercalciuria. One previous study demonstrated that most dogs with CaOx urolithiasis have hypercalciuria and higher blood ionized calcium relative to age-, sex-, and breed-matched controls without an obvious cause.[47]

Calcium balance is regulated by multiple organs including the parathyroid glands, kidneys, bones, and intestines. Though some cases of CaOx stones are associated with hyperparathyroidism, parathyroid hormone (PTH) concentrations have been reported to be normal in most people and dogs with CaOx stones.[46,54] A primary kidney defect is also considered unlikely as it is commonly associated with low to normal blood calcium and compensatory responses such as increased PTH release.[64,65] Bones serve as the major reserve of calcium in the human and canine body. Increased bone resorption has been repeatedly associated with urolithiasis in people.[52,53,66] This mechanism has not been evaluated in dogs.

Gastrointestinal calcium absorption is largely mediated by vitamin D. Vitamin D is absorbed from the diet in an inactive form and converted in the liver to 25-hydroxyvitamin D [25(OH)D]. The 25(OH)D form is then converted to the active form, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], which directly acts on the vitamin D receptor to increase calcium absorption from the diet among other effects. Transcellular and paracellular mechanisms of calcium absorption from the GI tract also exist but are not

mediated by vitamin D.[25,26] When 1,25$(OH)_2$D is no longer needed due to normalized calcium concentrations, 25(OH)D and 1,25$(OH)_2$D are converted to the relatively inactive metabolites 24,25-dihydroxyvitamin D [24,25$(OH)_2$D] and 1,24,25-trihydroxyvitamin D [1,24,25$(OH)_3$D] respectively in the liver (Figure 1). There are no commercially available assays for 1,24,25$(OH)_3$D so 24,25$(OH)_2$D is used as the marker of vitamin D inactivation.

A recent study in people found increased concentrations of 1,25$(OH)_2$D in patients with calcium kidney stones compared to controls.[54] The stone formers also had a higher ratio of 25(OH)D to the inactive 24,25$(OH)_2$D, and blood calcium was at the upper end of the reference range in the patients. These results suggest that greater activation and decreased relative deactivation of vitamin D may contribute to stone formation.

To our knowledge, only one study has reported vitamin D concentrations in dogs with CaOx urolithiasis. It was a small study in Miniature Schnauzers that compared 1,25$(OH)_2$D concentrations in 6 dogs with CaOx stones to 6 without stones.[46] No difference was found between the groups, but other vitamin D metabolites were not measured. Furthermore, most of the controls were younger than the average age of stone formation in dogs and could have been latent stone formers, and urine and blood calcium concentrations were compared to clinical healthy beagle dogs from a previous study.[67]

The objective of this study was to evaluate vitamin D metabolites (25(OH)D, 1,25$(OH)_2$D, and 24,25$(OH)_2$ D) in 20 dogs with a history of CaOx urolithiasis and in 20 age-, sex-, and breed-matched stone-free control dogs. Our hypothesis was that dogs with a history of CaOx urolithiasis have higher concentrations of 1,25$(OH)_2$D compared to

control dogs without urolithiasis. In addition, we hypothesized that the ratio of 25(OH)D to 24,25$(OH)_2$D is greater in stone formers.

Materials and Methods

Study Population

The study took place at the University of Minnesota Veterinary Medical Center (UMN VMC), and the population included dogs that had participated in genetic and metabolic studies on CaOx urolithiasis between February 2011 and November 2015.[68] Miniature Schnauzers, Bichons Frise, and Shih Tzus were selected to represent breeds where idiopathic hypercalciuria has been reported in CaOx stone formers.[47] Cases were defined as dogs with a history of uroliths comprised of ≥70% CaOx as determined by stone analysis at the Minnesota Urolith Center (polarized light microscopy and infrared spectroscopy). All cases had hypercalciuria, defined as a fasting urinary calcium-to-creatinine (UCa/Cr, mg/mg) ratio > .05 (the upper end of the 95% confidence interval reported in stone-free control dogs).[1] Controls had no history of urolithiasis and no evidence of urolithiasis on screening abdominal radiographs. All controls had a fasting UCa/Cr < .05. All dogs had a blood ionized calcium (iCa) and/or a serum total calcium concentration within laboratory reference ranges. The primary diet fed was obtained from the medical records.

Stored frozen serum samples (-80 C) were available from 61 dogs (26 cases and 35 controls) that met the criteria above. All samples had been obtained after food was withheld for 12-18 hours. Forty of the 61 samples (20 cases and 20 controls) were

selected for the present study with matching of breed, sex, and age (± 1 year) between cases and controls.

Vitamin D Assays

Serum samples were submitted to a Vitamin D External Quality Assessment Scheme-certified laboratory for analysis (Heartland Assays, Inc., Ames, IA). Three vitamin D metabolites were measured: $1,25(OH)_2D$ was measured using radioimmunoassay and $24,25(OH)_2D$ and 25(OH)D were measured using liquid chromatography-mass spectrometry.

Statistical Analysis

A Student t test was used to compare normally distributed data, including the mean age, ionized calcium concentration, $1,25(OH)_2D$, and 25(OH)D between case and control groups. Wilcoxon rank-sum tests were used for case-control group comparisons of data that did not follow a normal distribution, including UCa/Cr, $24,25(OH)_2D$, and the ratios of 25(OH)D/$24,25(OH)_2D$ and $1,25(OH)_2D$ /25(OH)D. A multivariable regression was also performed to determine the effects of sex, age, breed, diet, and stone status on the ratio of 25(OH)D/$24,25(OH)_2D$. A simple regression analysis was used to test the relationship between the ratio of 25(OH)D/$24,25(OH)_2D$ and log-transformed UCa/Cr (logUCa/Cr). All analyses were performed using R software for statistical computing (R, version 3.3.1. www.r-project.org) and a *P* value < .05 was considered significant.

Results

Forty dogs were included in the study. The cases and controls were breed and sex matched. Each group included 10 Miniature Schnauzers (5 male neutered and 5 female spayed), 5 Bichons Frise (3 male neutered and 2 female spayed), and 5 Shih Tzus (3 male neutered and 2 female spayed). The mean age of the cases (10.5 ± 2 years) at the time of sample collection was not significantly different from the mean age of the controls (9.8 ± 2.1 years, $P = .29$). In the case group, the mean age of first stone formation was 8.4 ± 2 years (range 4-11 years). Twelve of the 20 cases (60%) were recurrent stone formers with between 2-4 episodes of CaOx urolithiasis. Two diets were fed to three or more dogs. Both of these diets were therapeutic stone-prevention diets: diet A (Hill's Prescription Diet u/d Canine, Hill's Pet Nutrition Inc, Topeka, KS) and diet B (Royal Canin Veterinary Diet Urinary SO, Royal Canin USA, Inc. St. Charles, MO). Each was being fed to four case dogs. The remaining 32 dogs were fed 28 different diets; these diets were classified as "other" for the analyses.

Fasting urine calcium-to-creatinine ratios were measured in all dogs. Data are presented in Table 1. Hypercalciuria was a selection criterion for the cases, and as expected, they had a significantly higher UCa/Cr than the controls (Table 1, $P < .001$). Blood ionized calcium was measured in 33 dogs (17 cases and 16 controls). Blood ionized calcium was also significantly higher in the cases as compared to the controls ($P < .001$), although all measured ionized calcium concentrations were within the laboratory reference range.

Serum vitamin D metabolite data are presented in Table 1. The mean concentrations of 25(OH)D and $1,25(OH)_2D$ were higher in cases than controls, but the

difference did not reach statistical significance (*P* = .11 and .16 respectively). Median 24,25$(OH)_2$D concentrations were the same in both groups (*P* = .95). There was no significant difference in 1,25(OH)2D /25(OH)D between cases and controls.

Table 4: Calcium (blood and urine) and vitamin D metabolite analysis of the study population. For normally distributed data, mean is reported with ± SD; data that did not follow a normal distribution are reported as median (range).

Parameter	Controls	Cases	*P* value
iCa, mg/dL	5.2 ± .2	5.5 ± .2	**<.001**
UCa/Cr, mg/mg	.022 (.005 – .049)	.116 (.53 – .292)	**<.001**
25(OH)D, ng/mL	24 ± 13	42 ± 18	.11
1,25$(OH)_2$D, pg/mL	164 ± 69	198 ± 81	.16
24,25$(OH)_2$D, ng/mL	28 (11 – 40)	28 (4 – 40)	.95
25(OH)D/24,25$(OH)_2$D, ng/ng	1.17 (.98 – 1.58)	1.43 (.92 – 2.75)	**.012**
1,25$(OH)_2$D/25(OH)D, pg/ng	5.1 ± 3.2	5.1 ± 3.6	.92

P values in bold denote significance (*P* < .050).

When comparing ratios of vitamin D metabolites, the median 25(OH)D/24,25$(OH)_2$D was significantly higher in cases versus controls (*P* = .012). The ranges overlapped for the two groups, but 7 of the 20 cases (35%) had ratios above the highest ratio observed in the control group (Figure 2); these 7 highest ratios were present in 4 Miniature Schnauzers, 2 Shih Tzus, and 1 Bichon Frise. There was a moderate positive correlation (r= .35, *P* = .028) between 25(OH)D/24,25$(OH)_2$D and logUCa/Cr in the population as a whole. Further analysis revealed differences in this relationship between the groups. Within the control group, there was a strong positive correlation between 25(OH)D/24,25$(OH)_2$D and logUCa/Cr (r = .56, *P* = .011, Figure 3); in contrast,

the correlation within the case group was negative and approached but did not reach statistical significance (r = -.40, *P* = .081).

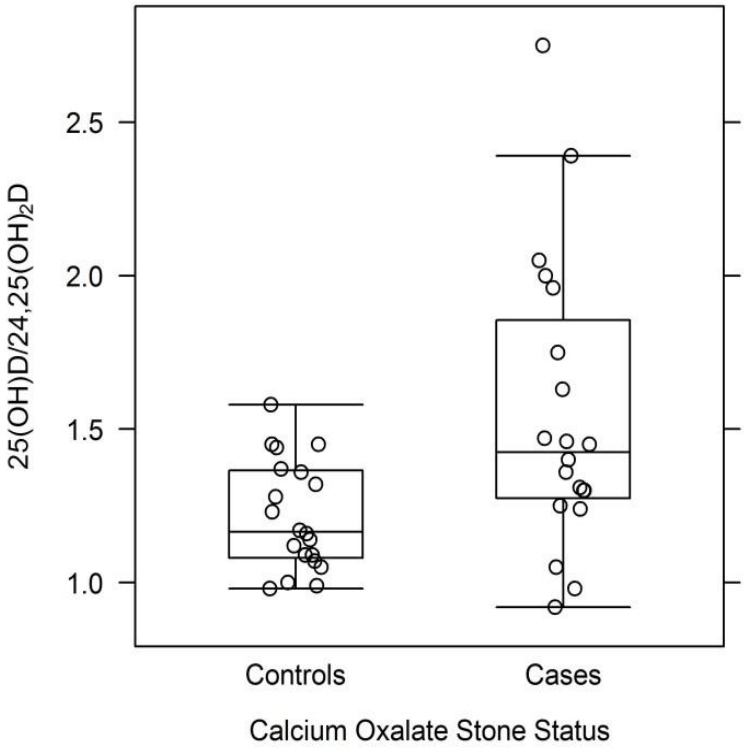

Figure 4: Box and whisker plots of 25(OH)D/24,25$(OH)_2$D in cases versus controls (P = .012). The boxes represent the 25th and 75th percentiles. The whiskers represent range of ratios. The dots represent each individual within the specified group.

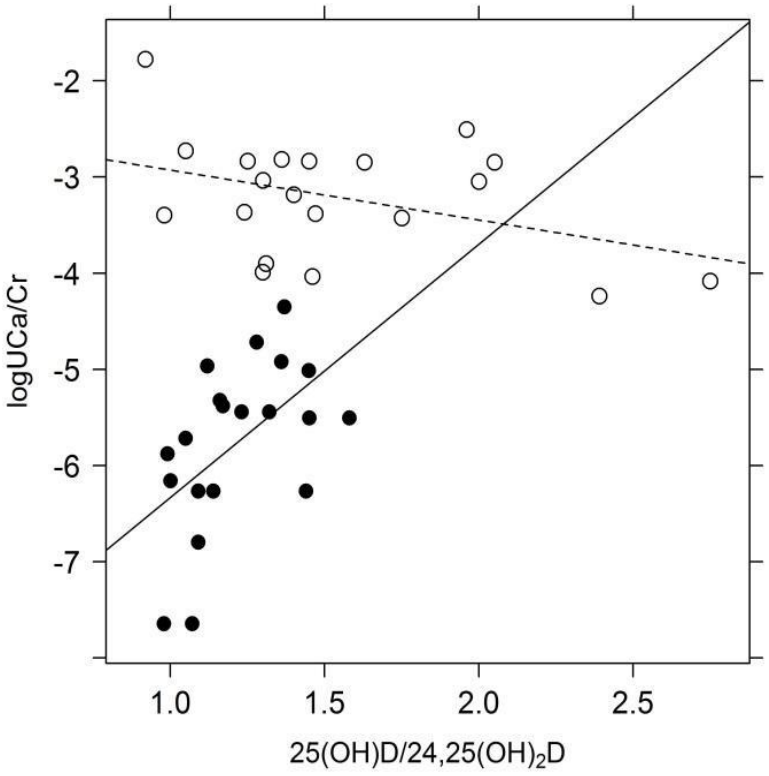

Figure 5: Relationship between 25(OH)D/24,25(OH)$_2$D and logUCa/Cr in cases (open circles, dotted line; r = -.40, P = .081) and controls (closed circles, solid line; r = .56, P = .011).

A multivariate analysis was performed to evaluate for the effects of sex, breed, age, and diet on 25(OH)D/24,25(OH)$_2$D (Table 2). CaOx stone status was the strongest predictor of 25(OH)D/24,25(OH)$_2$D (estimate = .44, *P* = .0010). Age also had a significant association (*P* = .023), but the effect was weak (estimate = .06). Diet approached significance as a predictor and was driven by a negative association between diet B and 25(OH)D/24,25(OH)$_2$D (estimate = -.43, *P* = .062).

Table 5: Multivariable regression model for the effects of age, sex, breed, diet, and CaOx stone status on the ratio of 25(OH)D/24,25$(OH)_2$D.

Parameter	Estimate	Standard Error	T value	*P* value
CaOx case	.44	.12	3.6	**.0010**
Age	.06	.03	2.4	**.023**
Sex (male)	-.13	.10	-1.3	.21
Breed				1.00
Miniature Schnauzer	Referent	Referent	Referent	NA
Bichon Frise	.01	.15	.05	.96
Shih Tzu	.00	.13	.03	.98
Diet				.052
Other	Referent	Referent	Referent	NA
Diet A	.14	.21	.65	.52
Diet B	-.43	.22	-1.9	.062

P values in bold denote significance (< .05).

Discussion

In this study, we found differences in vitamin D metabolites between dogs with CaOx urolithiasis and matched stone-free controls. Specifically, the cases had a greater ratio of 25(OH)D to a specific inactive less active vitamin D metabolite (25(OH)D/24,25$(OH)_2$D). Examination of the data showed that there was considerable overlap in this ratio between groups and that the difference was driven by a subset of stone forming dogs (7/20, 35%) with an increased ratio. This suggests that decreased conversion of 25(OH)D to 24,25$(OH)_2$D may contribute to CaOx stone risk in some but not all dogs.

Studies in people have shown alterations in vitamin D metabolism due to a deficiency in the CYP24A1 gene. This gene encodes the enzyme that converts 25(OH)D

and 1,25$(OH)_2$D to 24,25$(OH)_2$D and 1, 24,25$(OH)_3$D (Figure 1).[69] Loss of function of this gene leads to a relative increase in vitamin D metabolites with more vitamin D receptor activity as compared to vitamin D metabolites with less receptor activity. This results in higher than normal blood calcium and compensatory hypercalciuria. Approximately a third of the cases in our population showed similar ratio changes. However, since there is no data on ratios expected in dogs with CYP24A1 deficiency, we cannot determine if the degree of the change is consistent with this hereditary disorder. Given the lack of previous data, we also cannot be certain that the 25(OH)D/24,25$(OH)_2$D difference represents pathology within our case population or rather a protective mechanism in our control group. Of note, the ratios observed in cases varied not only across but also within breeds, suggesting that the pathophysiology of stone formation may differ even between individuals of the same breed.

Multiple metabolites of vitamin D were measured in the present study to completely evaluate the vitamin D status of the included dogs. While the only significant difference between cases and controls was the ratio 25(OH)D/24,25$(OH)_2$D, the median 25(OH)D and 1,25$(OH)_2$D were higher in cases versus control dogs. This study included a small number of dogs, and the interindividual variability was great. Furthers studies with larger sample sizes are needed to confirm whether there are differences between 25(OH)D and 1,25$(OH)_2$D in cases and controls.

There was a significant correlation between 25(OH)D/24,25$(OH)_2$D and UCa/Cr in control dogs but not in cases. All cases were selected to be hypercalciuric, which could eliminate the ability to detect a correlation. However, while all had UCaCr above the threshold of .05, there was variation within the groups; the UCa/Cr in cases ranged from

just above the highest value in controls to approximately 6 times it. This lack of correlation in the cases further demonstrates the multifactorial nature of hypercalciuria.

We also found a small significant positive association between the ratio and age. The estimate of this association was weak compared to the association between case status, however, it was significant. In humans, calcium deficiency has been reported in elderly populations which is thought to be secondary to several mechanisms including decreasing production of $1,25(OH)_2D$ by aging kidneys and decreasing sensitivity of the GI tract to $1,25(OH)_2D$, as well as several other molecular mechanisms.[70] It has also been proposed in human medicine that upregulation of CYP24A activity increase the conversion into $24,25(OH)_2D$. [71] These changes would be expected to decrease $25(OH)D/24,25(OH)_2D$. However, age-related changes in vitamin D metabolism have not yet been investigated in dog, and there could be species differences. A positive association with a higher ratio could be a result of increased 25(OH)D or decreased $24,25(OH)_2D$ due to alteration in vitamin D metabolism associated with increasing age.

A previous study showed significant variation in 25(OH)D between dogs being fed a variety of home cooked and commercially available.[39] Diet fed to each dog in this study was recorded, but, with the exception of urinary stone prevention diets, the numbers of each diet type were too low to draw any conclusions regarding impact on serum vitamin D metabolites in this study. There was a potential association between a lower ratio of $25(OH)D/24,25(OH)_2D$ and being fed a specific stone-prevention diet (Diet B) although this association was not present in a second stone-prevention diet (Diet A). Diet B has a lower vitamin D_3 content as compared to diet A (26.4 IU/100 kcal vs. 45.5

IU/100 kCal respectively for dry formulations). No data exists to further elucidate how these specific diets may impact vitamin D metabolism

There are a few limitations to this study. Only three breeds were included, and there were small numbers of dogs in each breed. Blood phosphorus and hormones involved in vitamin D regulation were not measured in present study including parathyroid hormone, and fibroblast growth factor 23. The control dogs were screened for stones using abdominal radiographs which are less sensitive than other modalities including contrast radiography or ultrasound for detecting small uroliths.[72] However, there were no significant outliers identified within the control population.

In conclusion, this data suggests that decreased conversion of 25(OH)D to the inactive 24,25$(OH)_2$D occurs in a subset of dogs with CaOx urolithiasis. Additional studies are needed to further evaluate how abnormalities in vitamin D metabolism contribute to stone risk in dogs. Further confirmation and characterization of these abnormalities may lead to development novel therapeutic targets in some dogs. Additionally, to our knowledge this is the first time that the ratios of 25(OH)D/24,25$(OH)_2$D and 1,25$(OH)_2$D/24,25$(OH)_2$D have been reported in dogs, and it may be useful in assessing vitamin D status in other canine diseases. The ratio of 1,25$(OH)_2$D to 1,24,25$(OH)_3$D may also provide further insight into vitamin D metabolism in future studies.

Chapter 4: Conclusions and Future Directions

This thesis has explored the challenges of measuring calcium in a clinical setting as well as the role of vitamin D metabolites in the development of CaOx urolithiasis. We have shown that there are thresholds of tCa that are strongly predictive of ionized hypercalcemia. This threshold is different depending on whether azotemia is present or not. This research was done on a heterogeneous population of hypercalcemic patients. The next steps in this project would be further characterization of these thresholds within different populations including hypercalcemia of malignancy versus other causes of hypercalcemia such as endocrine disease. It would also be clinically relevant to determine if such thresholds exist for hypocalcemia as well. And ultimately these thresholds need to be validated at additional laboratories.

We also determined that altered vitamin D metabolism may play a role in the pathogenesis of hypercalciuria and subsequent CaOx urolithiasis. The next steps to further characterize these alterations would be to evaluate vitamin D metabolites in a larger population of dogs with CaOx. It would also be important to evaluate fibroblast growth factor as well as parathyroid hormone in all these patients. The measurement of another vitamin D metabolite, $1,24,25(OH)_3D$ may also play a role in further characterizing metabolic abnormalities in this population. This test is not currently commercially available, but is under development.

It is likely that altered metabolism of vitamin D and its metabolites only affects a subset of CaOx urolith forming dogs so larger numbers are critical to establish its effect on risk for CaOx urolithiasis. The etiology of these abnormalities also remains

undetermined, and so further genetic evaluation may be warranted to look for specific underlying genetic defects within breeds.

In conclusion, total calcium concentrations above 12.0 mg/dL in non-azotemic dogs and above 14.0 mg/dL in azotemic dogs are highly predictive of ionized hypercalcemia (PPV= 95% and 94% respectively). However, it is important to note that there were patients that had ionized hypercalcemia with normal or only minimally increased total calcium. This highlights in continued importance of measuring ionized calcium when calcium disturbances8 are suspected. We also determined that altered vitamin D metabolism as characterized by decreased conversion of 25(OH)D to $24,25(OH)_2D$ may play a role in canine CaOx urolithiasis. Further research is needed to validate and further explore these findings.

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