Measurement of Pre and Postprandial Urine Calcium to Creatinine Ratio to Identify Calcium Oxalate Urolithiasis in Miniature Schnauzers

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Abstract:
The intent of this research is to identify a simple diagnostic test to detect abnormal calciuresis and predict calcium oxalate (CaOx) urolith presence in Miniature Schnauzers. We investigated the impact of postprandial time on the specificity of urine calcium:creatine (UCa/Cr) in identifying affected dogs. The hypotheses were: 1) Significant differences exist in fasted and postprandial UCa/Cr between urolith-forming and control schnauzers. 2) UCa/Cr increases significantly from fasted baseline at one or more postprandial time point(s).

Urine samples were collected from Miniature Schnauzers with (urolith-formers) and without (controls) CaOx uroliths in a fasted state and 1, 2, 4, and 8 hours after feeding a standardized diet. The change in UCa/Cr from baseline was calculated for each postprandial time. Urolithiasis status and the time point were assessed for impact on the UCa/Cr and change in UCa/Cr using a mixed model ANOVA. Based on 9 urolith-forming and 15 control dogs enrolled, urolith-forming Miniature Schnauzers have significantly higher mean UCa/Cr at 1 hour and 8 hours postprandial timepoints indicating altered calciuresis. The change in UCa/Cr was not significant at any post-prandial time point between or within groups.

This pilot study shows male urolith-forming Miniature Schnauzers have excessive calciuresis throughout the day, providing insight into the mechanism behind their formation of CaOx uroliths. If using the Ca/Cr ratio, the postprandial sampling time is not critical. This simple urine measurement has potential as a marker of urolith presence and possibly risk of urolith formation.
Measurement of Pre and Postprandial Urine Calcium to Creatinine Ratio to Identify Calcium Oxalate Urolithiasis in Miniature Schnauzers

Abstract (public):

Calcium oxalate urinary stones are a frequently encountered problem in Veterinary Medicine, as they have an increased incidence amongst several popular dog breeds; the Miniature Schnauzer, Yorkshire Terrier, Bichon Frise, Toy Poodle and Dachshund. These stones are a significant source of pain for affected dogs and financial strain for pet owners. The causes of calcium oxalate urinary stone formation are not fully known, but increased urinary calcium has been identified in affected dogs. We are quantifying calcium excretion by performing a measurement called urine calcium to creatinine ratio (UCa/Cr). The hypothesis of this research is that the UCa/Cr will be significantly greater in stone-forming dogs than non-stone forming dogs (controls). The second hypothesis is that the difference between the two groups will be even greater in the hours after feeding.

This findings of this study show that Miniature Schnauzers that form calcium oxalate urinary stones have significantly higher mean UCa/Cr than control dogs at multiple points throughout the day. The change in UCa:Ca over the day was not significant. The UCa/Cr proved to be a simply cost-effective biomarker to identify Miniature Schnauzers who may be at risk for CaOx urinary stone formation.
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1,25-dihydroxyvitamin D3 (calcitriol)
Calcium oxalate (CaOx)
Calcium sensing receptors (CaSR)
Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC)
Odds Ratio (OR)
Parathyroid hormone (PTH)
Relative supersaturation (RSS)
Renal outer medullary potassium channel (ROMK)
Tamm-Horsfall protein (THP)
Transient Receptor potential Vanilloid Channel type 5,6 (TRPV-5, TRPV-6)
Urinary Calcium to creatinine ratio (UCa/Cr)
Urine Specific Gravity (USG)
CHAPTER ONE

SECTION A: CALCIUM HOMEOSTASIS

Calcium homeostasis is tightly regulated by a number of pathways that are designed to maintain steady serum calcium concentration. Strict control ensures sufficient calcium for neuronal signaling, muscle contraction and many other biochemical pathways, whilst avoiding the deleterious effects of hypercalcemia such as dystrophic soft tissue mineralization. Calcium homeostasis is regulated by a number of mechanisms (see figure 1). The sources of serum calcium are intestinal absorption and the skeleton. This is balanced against calcium efflux, either excreted in urine or stool or stored as bone.

Although the skeleton is a vital component of calcium homeostasis as the reservoir of 99% of the body’s calcium, this thesis will only be discussing mechanisms of control of gastrointestinal absorption and renal elimination, reabsorption, and excretion.

1. Gastrointestinal absorption and elimination

Although calcium is absorbed throughout the intestinal tract, it mainly occurs mainly in the duodenum, ileum and cecum, and to a lesser extent in the proximal and distal colon. [1, 2]

There are three methods of calcium absorption across the gastrointestinal barrier: transcellular facilitated diffusion, transcellular vesicle transport and paracellular movement through tight junctions. Transcellular facilitated diffusion is a three step pathway through the enterocyte. In the initial step, calcium cations pass through the channels in the apical side of the enterocyte membrane (see Figure 2), along an electrochemical gradient. Evidence suggests that the TPRV6 channel (previously also referred to as ECaC2 and CaT1) is involved in the step, and other proteins and channels such as Ca,1.3 and TRPV5

Figure 1: Factors affecting calcium homeostasis
(also called ECaC1 and CaT2). The second step involves transport across the epithelial cell. The calcium cations bind to calbindin-D9k protein, which moves calcium across the cell to the basement membrane. The act of calcium cation removal from the apical side assists in maintaining the translumen-epithelial concentration gradient. Finally, calcium is transported across the basal membrane by one of two ways: either an ATP-ase pump or a Ca-Na exchange transporter.

The second mechanism of transcellular calcium movement is transcellular vesicle transport, which occurs via endocytosis-exocytosis transport. This is less frequently described, although has been demonstrated in chick models.[7]

The third method of calcium absorption across the intestinal barrier is paracellular movement through tight junctions. Tight junctions are regulated by various proteins, including claudins, which control which substances can pass through the junction. [8]

Figure 2: Pathways of gastrointestinal calcium absorption.

(A) Transcellular facilitated diffusion, which occurs by three steps: (1) movement through calcium channel such as TPRV6 on apical cell surface. (2) transport across cell by calcium binding protein, calbindin-D9k. (3) extrusion from basement membrane by either an ATPase pump or Ca-Na co-transporter.

(B) Transcellular vesicular transport,

(C) Paracellular movement through tight junctions.

Only a limited amount of ingested dietary calcium is absorbed across the intestine and the remainder is eliminated in feces. When serum calcium concentration is normal and there is abundant dietary calcium, the main route of calcium absorption is by paracellular diffusion down the concentration gradient. However, if additional calcium absorption is required, the demand is met mainly by the transcellular pathway as it has significant scope for upregulation, unlike the paracellular pathway that is saturatable.
In adult humans, it has been estimated that approximately 30% of dietary calcium is absorbed. However, this varies widely with reports of intestinal calcium absorption ranging from <10% to >60%. Fecal calcium is generally the primary means for calcium elimination as fecal calcium rises in parallel, when dietary calcium is doubled or tripled in humans and dogs. Conversely, when serum calcium is low, fractional intestinal absorption increases. This has also been demonstrated by a number of studies in dogs, which have been summarized elsewhere. This variability in fractional absorption is regulated by various mechanisms in an attempt to keep serum calcium at a constant concentration.

The most effective regulator of intestinal calcium absorption is the active form of vitamin D, 1,25-dihydroxyvitamin D3 (from here-on referred to as calcitriol). In response to hypocalcemia, parathyroid hormone (PTH) stimulates hydroxylation of the precursor 25-hydroxyvitamin D to calcitriol. Conversion requires 1-alpha-hydroxylase, an enzyme produced primarily by the kidney. It was previously considered that the cells of the proximal tubule were the main source of this enzyme, however it has now been demonstrated by immunohistochemistry and in situ hybridization that 1-alpha-hydroxylase is expressed, and hence calcitriol generated, throughout all segments of the nephron.

Calcitriol mainly circulates bound to a carrier protein: vitamin D binding protein. Calcitriol is lipophilic and thus can diffuse through the target cell’s membrane. Once in the cell, they bind to intracellular receptors in the nucleus called vitamin D receptors (VDR), and they alter gene transcription.

Although VDR are located throughout the body and have a variety of actions, only their effect on regulating calcium balance will be discussed here. When calcitriol activates the intestinal VDR, upregulation of the TRPV-6 gene causes increased expression of calcium epithelial channels. Activation of VDR also increases calbindin D9k production. Thus calcitriol increases calcium absorption from the intestinal tract.

Although the most significant action of calcitriol is to upregulate transcellular calcium movement, it also affects the paracellular absorption pathway. Calcitriol stimulates the VDR in the enterocyte to cause an upregulation of claudins-2 and -12. This increases the permeability of the tight junctions to calcium, thus allowing increased passive transport between cells.

Another regulatory pathway involves parathyroid hormone acting directly on the intestine. Using immunoreactive staining, some research has discovered PTH receptors located on intestinal epithelial cells. However, the specifics of this pathway have not been proven. Therefore, the main action by which PTH affects calcium absorption is indirect ie by increasing the conversion of Vitamin D to the active form calcitriol.

Other calcitropic hormones control absorption by causing a local effect on the intestine, or by influencing calcitriol, or both. Thyroid hormone appears to increase calcium absorption, possibly by increasing the Na-Ca efflux pump on the basolateral membrane, and/or increasing vesicular transport across the cell. Estrogen influences intestinal calcium absorption, and this effect is demonstrated by reduced intestinal calcium absorption in post-menopausal women. Estrogen appears to have genomic effects on the intestine to increase expression of TRPV5, TRPV6, calbindin-D9k and the plasma membrane calcium-ATPase pump. Prolactin has also been shown to directly increase intestinal calcium absorption, and is considered to be the principal calcitropic hormone in pregnancy and lactation.
hormone has a proliferative response on intestinal epithelium, and increases parathyroid gland activity. Growth hormone also appears to act directly on the intestine to increase calcium absorption, independent of calcitriol. [25]

A: 1.1: Role of Diet

The amount of calcium absorbed is affected by bioavailability of the calcium in the intestinal lumen, in addition to the endocrine factors mentioned above. Bioavailability is determined by the molecular structure and the substances bound to calcium. Calcium can be complexed in a way that renders it unavailable for absorption. Both ionic bonding and protein binding can be either promoted or inhibited by other intestinal contents.

Phytate is an example of ionic bonding inhibiting calcium absorption. Phytate has a strong negative charge and will bind to calcium cations in the intestinal lumen. This renders the complexed calcium poorly absorbable, as negatively charged phytate is repelled from negatively charged phospholipid cell membranes, including those of the gastrointestinal barrier. Hence diets high in phytates have impaired calcium absorption, as demonstrated by in vivo studies.[26, 27]

Dietary fiber influences calcium absorption in multiple ways. Prebiotics are fibers, typically soluble fibers like oligosaccharides, that stimulate growth or activity of beneficial gut bacteria and influence calcium absorption directly and indirectly.[28] Prebiotics promote the breakdown of phytate to indirectly enhance calcium absorption.[29] Prebiotics are rapidly fermented into organic acids which decreases luminal pH. Reduced gut pH promotes the dissociation of calcium salts, such as calcium carbonate, and subsequent absorption.[30-32] Prebiotics also promote calcium absorption by increasing permeability of tight epithelial tight junctions.[33-35] Furthermore, oral lactulose (0.9g/kg/day)[32] and oligofructose [31] increase gastrointestinal calcium absorption in dogs.

Oxalate is another example of ionic bonding decreasing the bioavailability of calcium. Oxalates are high in dietary products such as green leafy vegetables, and this renders the calcium effectively unabsorbable.[36] Intestinal oxalate concentration is affected by a number of factors, including microbes such as oxalobacter formigenes[37], however a more in depth discussion of regulation of intestinal oxalate is beyond the scope of this thesis.

Calcium intestinal absorption can be altered by the gastrointestinal microbiome. In cell culture models, calcium uptake was stimulated by the probiotic Lactobacillus Salivarius.[38] The mechanism of this pathway has not been confirmed.

Magnesium has an indirect action to decrease calcium absorption through the parathyroid-calcitriol pathway. Hypomagnesemia results in a functional hypoparathyroid state.[39, 40] In human medical literature, this phenomenon is referred to as magnesium-dependent vitamin D resistant rickets.

2. Renal handling:

Calcium in either the ionized or complexed form can pass freely through the glomerular basement membrane into the ultrafiltrate. However, most (98-99%) is resorbed from various segments of the nephron. Calcium transport across the renal tubular epithelium occurs by a similar process to that across the intestinal barrier; i.e. using paracellular and transcellular methods. The three-step transcellular pathway is similar to that described for intestinal absorption. Firstly, calcium enters the cell through a
calcium channel due to the electrochemical or electrical gradient. Secondly, calcium is transported to the basolateral membrane by a cytoplasmic binding protein, such as calbindin. Thirdly, calcium exits the cell across the basolateral membrane by either a Ca-ATPase pump or a sodium-calcium counter transporter.[41]

The predominate method and rate of absorption varies among the different segments of the nephron.[42] Approximately 60-65% of calcium resorption occurs in the proximal tubule, mainly by paracellular processes.[43] Solute drag occurs as a significant proportion of fluid passes out of the ultrafiltrate into the interstitial space, driven mainly by a sodium concentration gradient. As calcium ions are dissolved in this fluid, they are carried out of the tubular lumen, between the epithelial cells, and into the interstitial space. Calcium and sodium ions are able to freely pass between cells due to cation-permeable channels formed by claudins 16 and 19. [44] Calcium excretion in the proximal tubule is predominately determined by filtered load; a fractional excretion study in the dog demonstrates decreased filtered calcium in volume-depleted dogs and increased filtered calcium in saline-loaded dogs.[43]

No significant calcium transport occurs in the thin loop of Henle. The thick loop of Henle resorbs approximately 10-30% of the reclaimed calcium, predominately through paracellular diffusion. In this segment, paracellular diffusion is driven by the lumen positive transepithelial potential. This electrical gradient is maintained due to the apical Na-K-2Cl cotransporter and the renal outer medullary potassium channel (ROMK)[45] (see Figure 3). Due to the calcium ion’s positive charge, paracellular movement out of the lumen is promoted.

In the distal tubule and collecting duct, calcium resorption is primarily by a transcellular route. There is some variation in the intracellular binding proteins that assist with transcellular movement. Parvalbumin is the predominate intracytoplasmic binding protein in the early distal tubule, while calbindin-D28k is the predominate transporter in the late distal tubule and connecting tubule, although Calbindin-D9k is also expressed[46],[47]. This is different from transcellular intestinal transport described above; calbindin-D9K is the only calbindin that is expressed in mammalian intestinal cells.

Calcium resorption is controlled by several homeostatic mechanisms. The requirement to adjust calcium movement in response to demand is primarily met by adjusting the transcellular movement in the distal nephron, although resorption in other parts can also be adjusted. PTH acts directly on the thick

![Figure 3. The lumen-positive trans-epithelial gradient is driven by the Na-K-2Cl co-transporter and the ROMK channels. This promotes paracellular diffusion in the thick loop of Henle.](image)
ascending loop of Henle, distal tubule and collecting tubule to cause up-regulation of the calcium channel TRPV 5 and increased expression of calcium binding proteins.[48]

Calcitriol increases calcium resorption, although not to the same extent as PTH. Increased calcitriol concentrations increase expression of calbindin-D9K in the proximal tubule, loop of Henle and the distal tubule epithelial cells.[49] Calcitriol does not change calbindin-D28k expression as this protein appears to be vitamin D independent.[50] Calcitriol also has a weak action on increasing paracellular calcium movement.

The thick loop of Henle has calcium sensing receptors (CaSR) in the basolateral membrane, allowing local autocrine control. Increased activation of CaSR, for example by hypercalcemia, cause inhibition of the TRPV5 channel on the luminal membrane and reduces transcellular resorption. CaSR also inhibit activity of the Na-K-2Cl co-transporter and the ROMK channel, which dissipates the transepithelial gradient driving paracellular transport.[51]

**A:2.1: Drugs and diseases affecting calciuresis:**

There are various drugs that incude calciuresis by inhibition of the Na-K-2Cl cotransporter, although the physiology of the resulting calciuresis depends upon which segment of the nephron is affected. Furosemide inhibits the Na-K-2Cl cotransporter in the thick loop of Henle. This leads to a loss of the transluminal voltage gradient, without which there is no driving force for paracellular calcium resorption.[52] This results in hypercalciuresis. Thiazide diuretics, such as hydrochlorothiazide, inhibit the Na-K-2Cl cotransporter in the distal nephron. This diuretic effect in the distal tubule results in a decreased extracellular volume. This prompts increased passive resorption of sodium through upregulation of the sodium-hydrogen exchanger in the proximal nephron. As a result passive solute drag is increased, resulting in increased calcium resorption.[53, 54]

Primary hyperaldosteronism causes hypercalciuria and is described as a risk factor for calcium nephrolithiasis in humans.[55] The volume expansion from primary hyperaldosteronism decreases sodium resorption in the proximal tubule and hence reduced calcium resorption occurs.

Hyperadrenocorticism is another disease which induces hypercalciuria[56] and is also a risk factor for the formation of canine calcium oxalate urolithiasis.[57] The proposed mechanism is excessive cortisol acting as a ligand for the mineralocorticoid receptor.[58] Activation of mineralocorticoid receptors cause fluid retention and hypercalciuresis by the same aldosterone-mediated mechanism mentioned above. The mineralocorticoid receptor in the distal tubule is also activated if local cortisol is not effectively metabolized to cortisone.[59] This can occur due to a deficiency in 11b-hydroxysteroid dehydrogenase, an inherited human condition named “apparent mineralocorticoid excess” or pseudohyperaldosteronism. As result of hypercalciuresis, nephrocalcinosis is a common feature of this disease.[60]

A similar mechanism can be induced by some exogenous glucocorticoids. Corticosterone and dexamethasone were also demonstrated to also activate mineralocorticoid receptors to varying degrees in the canine brain.[61]

Bisphosphonates are used to alter calciuresis. Traditionally used to inhibit bone resorption for treatment of osteoporosis, they are now being suggested for use in human patients with idiopathic hypercalciuria to decrease mobilization of skeletal calcium, and subsequent excretion.[62]
Diseases causing calciuresis were first classified by Pak et al [63] based on the different predominant underlying mechanism: absorptive hypercalciurias, resorptive hypercalciurias and renal hypercalciurias.

Absorptive hypercalciurias are due to hyperabsorption from the gastrointestinal system. In humans absorptive hypercalciuric conditions are sometimes diagnosed by an oral calcium-loading test which results in a great increase in urinary calcium. This type of hypercalciuria can be resolved with a low calcium diet. A disease model of this is mice with overexpression of TRPV-6 gene developing calciuresis.[64] Exogenous vitamin D excess or diseases causing increased endogenous calcitriol production also result in enhanced gastrointestinal absorption. These diseases appear to be only described in human medicine and include granulomatous disease, most notably sarcoidosis.[65] Other diseases include infectious granulomatous disease such as histoplasmosis[66], coccidomycosis[67] and bartonellosis[68]. Possibly granulomatous disease in animals which results in overproduction of calcitriol may cause a similar response.

Resorptive hypercalciuria refers to conditions that stimulate increased bone resorption. Hyperparathyroidism is the most common example of this. Hyperparathyroidism causes hypercalcemia which results in hypercalciuria; if there is increased calcium in the serum this will translate into increased calcium in the ultrafiltrate. Despite the direct effect of PTH on the distal tubules in upregulating calcium resorption, hypercalciuria overpowers the capacity for tubular resorption. The clinical result of hypercalciuresis from hyperparathyroidism is demonstrated in a retrospective evaluation of canine hyperparathyroid patients, 65/210 hyperparathyroid dogs had concurrent calcium based urolithiasis.[69] Further evidence for this causal relationship is seen in post-parathyroidectomy human patients who have resolution of the calciuresis and associated decreased likelihood of calcium nephrolithiasis after surgery.[70]

Renal hypercalciuria is also known as renal leak hypercalciuria or diet-independent hypercalciuria. Patients with primary renal hypercalciuria always have high urinary calcium, regardless of fasting state or diet. Therefore, only a moderate increase in urinary calcium occurs with the calcium-loading test. A variety of conditions affect the ability of the tubules to resorb calcium. Two conditions discussed earlier, hyperadrenocorticism and hyperaldosteronism, are examples of renal hypercalciuria affecting veterinary patients.

There are several additional examples of renal hypercalciuric which have been described in people, but not in dogs. Some of the various forms of Bartter syndrome result in renal hypercalciuria. Bartter syndrome type 1 is a genetic mutation in the SLC12A1 gene, which causes dysfunction of the Na-K-2Cl cotransporter on the basolateral membrane of the thick ascending loop of Henle. This causes loss of the transluminal electrochemical gradient that is responsible for driving sodium, water and electrolyte resorption similar to furosemide’s mechanism of action. Marked hypercalciuria, due to the loss of paracellular solute drag, generally results in medullary nephrocalcinosis in the first few months of life.[71]

Another genetic disease is “familial hypomagnesemia with hypercalciuria and nephrocalcinosis” (FHHNC). This mutation results in defective expression of claudins 16 and 19, which prevents development of cation-selective paracellular channels and removes the transepithelial gradient required for paracellular calcium resorption.[44]
In patients with primary renal hypercalciuria and unidentified underlying cause, the hypercalciuria is referred to as idiopathic. This is thought to have both a genetic and environmental cause due to over-representation in certain ethnicities and geographic areas such as amongst the Italian population.[72]

Bataille et al[73] investigated 42 hypercalciuric stone forming human patients and only identified one with primary renal hypercalciuria. It could be suggested that primary bone resorption is more common cause of hypercalciuria in people compared with renal hypercalciuria, but this hypothesis is limited by the small sample size. No similar data is available in dogs. With the exception of dogs with hypercalcemia or hyperadrenocorticism, the underlying cause of canine urolithiasis is usually not identified.
SECTION B: KINETICS OF CALCIUM OXALATE CRYSTAL FORMATION AND GROWTH

The precipitation of crystals and formation of uroliths depends upon urinary concentration of calcium oxalate and other relevant mediators.

1. Urine supersaturation
Relative supersaturation (RSS) is widely used as an assessment of risk of a specific stone formation. Briefly, the concentrations of calculogenic substances calcium, magnesium, sodium, potassium, ammonium, phosphate, oxalate, citrate, sulfate, uric acid and chloride are required, and calculations are made regarding the possible complexes that can be formed. The scale is adjusted so an RSS of 1 is equal to a concentration at which the salt is just soluble. Hence an RSS < 1 is termed undersaturated and an RSS > 1 is said to be supersaturated. A supersaturated solution can be said to be in a precipitation or nucleation zone when precipitation or amorphous aggregation occurs. Alternatively a supersaturated solution may be in a metastable state, when spontaneous precipitation does not occur.

Relative supersaturation was found to be significantly higher in 17 dogs with CaOx urolithiasis when compared to 17 control dogs.[74] The change in RSS is often used as a marker to assess the effectiveness of intervention measures designed to reduce calcium excretion or urine supersaturation in healthy dogs. For example, feeding healthy beagles a sodium chloride supplement, in addition to a maintenance urinary diet, significantly decreased their urine oxalate concentration and the relative urine supersaturation.[75] The main advantage of using RSS to estimate stone formation risk is that the measurement is rapidly available to researchers. Measuring clinical stone formation would take years of research and be far more cost and labor intensive.

However, there are a number of limitations in extrapolating RSS to clinical calcium oxalate (CaOx) stone formation risk. First, RSS does not account for all components involved in urolith formation. Many other proteins that are not included in the RSS have also been identified in CaOx uroliths. One study of human CaOx identified 68 other proteins, including inflammatory proteins such as immunoglobulins, complement C3a and fibrinogen were involved.[76] Second, urolith formation is influenced by more than simple urinary mineral concentration. Other properties of urine account for the existence of a metastable state in which the urine can hold large amounts of solutes beyond saturation level. These properties that inhibit urolith growth will be discussed below. Third, RSS does not take into account the environment in which CaOx uroliths may be initiated. RSS measurement is that it is typically measured on urine from a voided sample or collected directly from the bladder. This measurement does not represent the RSS of the ultrafiltrate in the kidney, where the initial crystallization may form.[77] Furthermore, RSS of the ultrafiltrate varies at different sites in the nephron, due to the changing concentrations of calculogenic substances and water.[78] If RSS in the loop of Henle causes nucleation, then this precipitation may be responsible for further growth, regardless of the final urine RSS. Although occasional micropuncture studies[78] overcome this limitation, the majority of studies reporting changes to RSS are possibly an oversimplification of a more complex process.

2. Promoters and inhibitors of crystallization, aggregation and growth
Stone development is a multi-step process. In humans, this is described as (a) nucleation, (b) aggregation and growth of crystal formation, (c) cell-crystal interaction with movement into the interstitium, (d) crystal retention and attachment.[79]
In human kidneys, it is accepted that calcium oxalate crystal aggregation often occurs secondary to formation of a nucleus of Randall’s plaque. Randall’s plaques are mineral depositions in the renal papilla, which serve as a nidus for further crystal formation. They are divided into type I and type II by the histological location of calcium accumulation along the nephron. Type I Randall’s plaques appear to be more common, occur in human idiopathic-calcium-stone formers, and are likely related to hypercalciuria.[80, 81] This process starts with calcium phosphate deposits at the basement membrane in the thin loop of Henle. It is suggested this might occur due to increased post prandial calcium absorption at the thick loop of Henle, which is then carried down the medulla by the vasa recta.[80] Alternatively there may be defective calcium resorption in the proximal tubule, when results in an increased luminal calcium load being delivered to the thick loop of Henle.[82] Regardless of the origin of the calcium, the plaque accumulates in the interstitium, eventually penetrating the epithelium or becoming exposed when there is other urothelial damage. It has been hypothesized that oxidative epithelial damage from inflammatory mediators in metabolic syndrome may be responsible.[83, 84] Interestingly, calcium oxalate nephrolithiasis is associated with metabolic syndrome, high triglyceride and high cholesterol concentrations.[85] This leads to speculation that familial hypertriglyceridemia of Miniature Schnauzers may have a role to play in renal tubule damage and canine nephrolith pathogenesis.

Once the epithelium is eroded, the exposed calcium deposits culminates in crystal aggregation and stone attachment at the renal papillae.[77, 80] From here there is further growth into nephroliths or inhibition of crystal formation depending on urine constituents or the “urinary milieu” as further discussed below.

There are variations in distributions of Randall’s plaques. Type II plaques cause calcium deposits inside the tubule lumen of collecting ducts, also known at ducts of Bellini, in obese patients receiving intestinal bypass.[80] This type of plaque does not appear to be associated with hypercalciuria[80], so is perhaps unlikely to be a useful model for hypercalciuric miniature schnauzers.

Urocystoliths occur rarely in humans compared to dogs. The above process of human nephrolith formation is extrapolated to canine urolith formation, although there are inherent limitations based on the current available literature. First, it has not been established if Randall’s plaques are involved in the pathogenesis of canine nephroliths. Therefore, the same nucleation, aggregation, retention, attachment model may not apply. Second, factors causing the growth of nephroliths have been extrapolated to factors which cause clinical disease in dogs, which predominately involves urocystoliths. Although it is possible there are differences in growth of urocystoliths compared to nephroliths, the basic concepts of urolith growth and aggregation will be discussed.

**B: 2.1 Promotors**

A promotor is a substance or an environment that makes crystal formation and/or growth more likely. Chemicals other than calcium and oxalate can promote CaOx crystal formation by increasing RSS. In vitro addition of sodium urate [86] and cystine [87] to human urine have been shown to promote crystallization of calcium oxalate. Although the mechanism was once considered to be due to epitaxy (a crystalline overlayer on a crystalline substrate with a similar orientation[88]) this theory has now been largely disproven.[87, 89] Rather, urate and cysteine action is referred to “salting out” CaOx due to increasing RSS to a supersaturated state.[87, 89]
Another chemical group which can act as a promotor is calcium salts. Calcium carbonate crystals act as promotors of calcium oxalate growth by providing a nidus for lithogenesis.[90] Further growth is promoted by epitaxy.[90] Calcium phosphate can also act as a nidus promoting calcium oxalate growth. A rather extreme example is a case report of ectopic bone (calcium phosphate) formation in a feline bladder, promoting CaOx urolithiasis.[91]

Acidic urine can be a promoter of crystalline growth. This occurs by two mechanisms. First, the solubility of calcium oxalate decreases in slightly acidic solutions. In one veterinary study, the calcium oxalate saturation of feline urine was lower when the urine pH was above 7.2 and higher when the urine pH was below 6.5.[92] Second, acidic urine reduces the effectiveness of both organic and inorganic inhibitors. In human studies, research shows macromolecule inhibitors (discussed below) are less effective in acidic conditions than at neutral or alkaline pH.[93] In addition, acidic pH increases resorption of citrate in the proximal tubule so there is less available to form complexes with calcium.[94, 95]

B: 2.2 Inhibitors

Inhibitors are polyanionic substances that retard the development and/or growth of calcium salt crystals. They can be inorganic or organic substances. The inorganic fraction is composed predominately of crystalloid complexors. Organic inhibitory compounds include urinary macromolecules, crystal surface binding substances, and components of non-crystalline stone matrix.

B: 2.2.1: Inorganic inhibitors: crystalloid complexors

Inorganic cations and anions inhibit stone formation by crystalloid complexing. Cations (primarily magnesium) bind to oxalate and anions (mainly citrate and pyrophosphate) bind to calcium. This reduces the urinary concentration of free calculogenic ions. This may result in an increase in the metastable limit as was demonstrated with pyrophosphate.[96]

Citrate is sometimes referred to as a crystalloid complexor, as it forms complexes with calcium. However, the relevant calcium chelation occurs in the gastrointestinal tract and in circulation. The main action of urinary citrate is now thought to be direct inhibitory action on the calcium oxalate crystal surface, rather than chelating urinary calcium.[97] Urinary citrate was demonstrated to have some importance in inhibiting stone formation as one study has identified hypocitraturia in >50% of human nephrolithiasis patients.[98]

B: 2.2.2: Organic inhibitors: Urinary Macromolecules and Crystal Surface Binding Substances

Organic inhibitors include urinary macromolecules which bind to the crystal surface and the organic matrix within the crystal. Some substances may occur both dissolved in urine and within the matrix.

Urinary macromolecule inhibitors are glycoproteins that covering the crystal surface and act as a biofilm. The hydrophilic portion attaches to the crystal, and exposes the hydrophobic portion to surrounding urine or ultrafiltrate. The altered hydrophobic crystal surface has only a weak electrical charge and is therefore not an attractive binding site for urinary ions.

Nephrocalcin is an inhibitory urinary macromolecule that may have a role in protection against urolithiasis. This glycoprotein is observed in different forms in urine of humans with and without a
history of CaOx urolithiasis. Nephrocalcin type A and B are strong inhibitors of crystalline growth. Reduced quantities of these were found in some stone forming humans [99] and possibly in dogs.[100] Rather, affected patients had greater concentrations of nephrocalcin type C and D, which act as only weak inhibitors.

Other macromolecules include other protoglycans such as Tamm-Horsfall protein (THP) and bikunin, and urinary prothrombin fragment 1.

Further proof of the importance of inhibitors is demonstrated by knockout mice models of two other glycoprotein inhibitors; ostoeopontin and THP.[101] Knockout mice without ostoeopontin, TPH or without either of these can be induced to form CaOx crystalluria when hyperoxaluria was induced. The wild type mice with normal concentrations of ostoeopontin and THP did not form these crystals.[101]

**B: 2.2.3: Organic inhibitors: the soluble stone matrix**

In addition to the crystalline components of a urolith, a lattice of organic material, called the matrix, is present. This organic matrix is difficult to quantify, as it is destroyed by quantitative stone analysis laboratory techniques. The matrix arises from urine constituents – both those present in urine at the beginning of stone formation and those produced by trauma from irritation and inflammation during stone aggregation and growth. In healthy humans, heparin sulfate, the key GAG component, strongly inhibits crystal aggregation and growth.[102] However, the role of heparin sulfate in canine urolithiasis has not been evaluated.
SECTION C: CLINICAL CALCICUM OXALATE UROLITHIASIS IN DOGS

1. Introduction
CaOx urolithiasis may result in clinical disease such as infection, urinary obstruction or pain in dogs. The consequences of urolithiasis depend, in part, upon the location within the urinary system affected. Certain breeds have a strong predisposition to CaOx urolithiasis, which can lead to further studies into the underlying cause. The popularity of these predisposed breeds with the American public makes research into the pathogenesis of CaOx urolithiasis an important priority.

Laboratory submissions of CaOx uroliths removed from dogs have been increasing in frequency. In the first year of operation in 1981, the Minnosota Urolith Center reported only 5% of canine uroliths were CaOx based.[103] This is compared to canine urolith submissions analyzed during 2007, of which 41% were CaOx.[103] A similar distribution and trend was noted at the Urinary Stone Analysis Laboratory based in Davis, California.[104] A contributing factor is suspected to be a decline in struvite submissions. One relevant point to note is that this data represents stones submitted for analysis, not the incidence of clinical disease. The recommended treatment for suspected struvite stones is now dissolution with dietary and anti-microbial treatments, rather than stone retrieval methods, so a decrease in struvite uroliths submitted for analysis is expected.

2. Predisposing factors
Causal risk factors for CaOx urolithiasis are largely unknown. Epidemiological studies have identified various dog factors listed below which are associated with an increased odds ratio of disease. However, the mechanism or pathogenesis has not been identified. These factors do not induce disease in all dogs, and correcting them has not been demonstrated to reverse the increased risk of disease or recurrence.

A potential limitation of a discussion on risk factors is that multiple epidemiological studies use data from Minnesota Urolith Center, sometimes reporting from the same or overlapping time periods. This can lead to overrepresentation of the data in the literature.

2.1. Urine factors
2.1.1. Calcium and oxalate concentration: Hypercalciuresis is a risk factors for canine calcium oxalate urolithiasis.[105, 106] Unlike urine calcium, urinary oxalate concentration is not considered of primary importance for calcium oxalate stone formation in dogs.[105-107]

2.1.2. Other urine constituents: Dogs with abnormal concentration of any of the crystalline inhibitors or promoters discussed in Section B are at increased risk for calcium oxalate urolithiasis.

2.2. Patient factors
2.2.1. Gender: An increased risk for male dogs was identified in some studies. [104, 108-110] It is possible that calcium oxalate urolithiasis is identified more commonly in male dogs because they more frequently develop urethral obstruction. Another suggested reason is an estrogen-dependent increase in urinary citrate, resulting in a decrease in urinary calcium and oxalate. This has been demonstrated in human studies involving estrogen supplementation. [111] However the majority of female dogs which received veterinary care (and hence may have urolithiasis diagnosed) are spayed, with little estrogen production so this mechanism may not be as significant in veterinary patients.
2.2.2. *Breed:* Certain breeds are significantly over-represented and summarized in the table below. It has been suggested that some breeds may have idiopathic hypercalciuresis.[106]

<table>
<thead>
<tr>
<th>Breed</th>
<th>Odds Ratio (OR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Schnauzer</td>
<td>18.1[109]</td>
</tr>
<tr>
<td>Miniature Schnauzer</td>
<td>14.1[109]-21.6[104]</td>
</tr>
<tr>
<td>Lhasa Apso</td>
<td>10.1[104]-10.9[109]</td>
</tr>
<tr>
<td>Jack Russell Terrier</td>
<td>9.9[109]</td>
</tr>
<tr>
<td>Papillion</td>
<td>9.9[109]</td>
</tr>
<tr>
<td>Bichon Frise</td>
<td>6.6[109]-23.6[104]</td>
</tr>
<tr>
<td>Yorkshire Terrier</td>
<td>6.5[104]</td>
</tr>
<tr>
<td>Keeshond</td>
<td>4.6[104]-5.5[109]</td>
</tr>
<tr>
<td>Pomeranian</td>
<td>4.9[109]-7.2[104]</td>
</tr>
<tr>
<td>Samoyed</td>
<td>4.6[109]</td>
</tr>
<tr>
<td>Shih Zhu</td>
<td>4.5[109]-10.2[104]</td>
</tr>
<tr>
<td>Chihuahua</td>
<td>3.9[109]</td>
</tr>
<tr>
<td>Cairn Terrier</td>
<td>3.7[109]-7.3[104]</td>
</tr>
<tr>
<td>Maltese</td>
<td>3.5[109]-5.2[104]</td>
</tr>
<tr>
<td>Miniature and Toy Poodles</td>
<td>3.3[109]</td>
</tr>
<tr>
<td>West Highland White Terrier</td>
<td>3.3[109]</td>
</tr>
<tr>
<td>Dachshund</td>
<td>2.7[109]</td>
</tr>
</tbody>
</table>

Dogs of non-urolith forming breeds, such as retrievers, greyhounds and Pitbulls, have been found to have significantly greater colonization of *Oxalobacter formigenes* when compared to urolith-forming dogs[37]. This suggests that it may be one breed-associated patient-related risk factor. There are likely to be many other breed-related differences which have not yet been identified.

2.2.3. *Age:* Dogs over 4 years of age are at least 5 times more likely to form calcium oxalate uroliths.[109] The mean age of dogs at first time stone removal was 8 years with a standard deviation of 3 years.[108]

2.2.4. *Body Condition Score:* It has been suggested that obesity is associated with calcium oxalate stone formation and/or calcium oxalate crystalluria[112], however the causal relationship has not been proven. It has been hypothesized that obesity is likely to cause urine acidification, however no difference in urine pH was observed between groups in the aforementioned study.

2.2.5. *Body Size:* Smaller breed size is associated with corresponding increased risk of disease[110]. One hypothesis for this increased risk is small breed dogs have a greater surface area and have a higher energy requirement per kilogram body weight. A higher energy requirement will translate into greater mineral intake if the diet is kept constant. However canine athletes, such as sled dogs, who have a significantly greater calorie requirement do not have a higher prevalence of CaOx urolithiasis, so this hypothesis is uncertain.

2.2.6. *Concurrent disease:* Diseases which cause hypercalciuresis were discussed in Section A, and these increase the patient’s risk. The two most significant diseases are likely to be hyperadrenocorticism which increases the odds ratio by a factor of 10 [57] and primary hyperparathyroidism.
3. **Diagnosis and treatment**

Calcium oxalate uroliths are visible on radiographs as smooth or spiky, radio-opaque, mineral dense structures. Typically they appear as many small round stones with a diameter approximately 2-7mm.[113] Although 2mm is often considered the smallest size calculi that are radiographically detectable, one paper found calcium oxalate urocystoliths 1mm and smaller were only missed on 2-5% of survey films using a simulated bladder model.[114] These characteristics can be used to help identify CaOx uroliths from uroliths of other types. Contrast radiography tends to be less useful for CaOx detection as calcium based urocystoliths tend to be isopaque to iodinated contrast.[114]

Ultrasound can also be used to identify uroliths. Ultrasonography was found to be superior to radiography in detecting small human nephroliths[115], and it could be speculated that the same is true for either canine nephroliths and possibly canine cystoliths. On a phantom bladder model with 7.5MHz transducer, ultrasonographic assessment was slightly superior to survey radiographs, with an average sensitivity of ~97% considering all uroliths types and sizes, including <1mm.[114] Limitations of ultrasonography is that it consistently overestimated the size of nephroliths and had low specificity.[116] These restrictions may or may not apply to canine cystoliths. The frequency of the transducer will affect the sensitivity of detection.[114]

The use of computer tomography to detect urolithiasis presence and identify stone type has been uncommonly described in dogs.[117] Although this has high accuracy for identifying calcium oxalate type stones in 93-96% simulated models, the additional cost, sedation and radiation exposure may not be justified in the majority of dogs.

Once uroliths are observed, identification of any of the above risk factors (signalment, urine pH, calcium oxalate crystalluria, concurrent predisposing disease or medications) can be useful to predict calcium oxalate urolith composition. Mineral type could be predicted in approximately 70% of urocystoliths using an algorithm based on signalment alone.[113] Ultimately, calcium oxalate urolithiasis is confirmed by submission of a urolith for quantitative mineral analysis.

Treatment for calcium oxalate urolithiasis is removal by surgery or minimally invasive methods. Indications for removal are the presence of clinical signs or dysfunction related to urolithiasis. In addition, urocystoliths that are small enough to fit into the urethra and judged at risk of causing future lower urinary tract obstruction are also good candidates for removal. These signs or dysfunction may present as stranguria, pollakiuria or urinary obstruction from urocystoliths, urethroliths and/or ureteroliths. Upper urinary calculi are generally asymptomatic. However they occasionally may result in pain and/or renal dysfunction from outflow obstruction, which then warrants their removal.

In general, nephroliths are not routinely removed. The majority of the time their presence appears to be of little clinical consequences and dogs are generally asymptomatic. ACVIM recommendations suggest only considering removal if there are clinical signs such as urinary obstruction, persistent infection or pain. Minimally invasive options include breakdown by extracorporal shock wave lithotripsy or percutaneous nephrolithotomy. Other more invasive options for removal are pyelotomy or nephrectomy.[118]

Methods for urocystolith and/or urethrolith removal are summarized below. Patient urethral size, urolith size and number, and equipment will dictate the methods available.
<table>
<thead>
<tr>
<th>Method</th>
<th>Indications</th>
<th>Limitations and potential complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural voiding</td>
<td>Suitable for very small stones (1-2mm).</td>
<td>Requires the urethral lumen to accommodate at least an 8Fr catheter size to be effective.</td>
</tr>
<tr>
<td>Catheter retrieval</td>
<td>Suitable for very small stones as above.</td>
<td></td>
</tr>
<tr>
<td>Voiding urohydropulsion</td>
<td>Suitable for stones up to 5-7mm depending on size of urethra[119].</td>
<td>Low but present risk of bladder rupture with over-distention, inadequate anesthesia or preexisting weakness.</td>
</tr>
<tr>
<td>Cystoscopic basket removal</td>
<td>Suitable for very small stones (1-3mm)</td>
<td>Suggested size limit of less than 4mm in female dogs and less than 2-3mm in most male dogs.[120]</td>
</tr>
<tr>
<td>Lithotripsy</td>
<td>Can be combined with cystoscopic basket removal or voiding hydropulsion to remove fragments[121].</td>
<td>Lithotripsy may be more time consuming for male dogs than a cystotomy, depending on size and number of stones present[122]. Risk of bladder rupture as for voiding hydropulsion, also damage from cystoscope or laser fiber[122] and transient urethral swelling [121]. Low risk of stones remaining after procedure (complete stone removed in 82-87% of cases[121-123]).</td>
</tr>
<tr>
<td>Retrograde hydropulsion</td>
<td>Relocates urethroliths into the bladder for removal by cystotomy.</td>
<td></td>
</tr>
<tr>
<td>Cystotomy</td>
<td>Suitable for any number and all sizes of stones.</td>
<td>Risks associated with wound healing. Incomplete removal of stones in 15-20% of cystotomy cases.[124]</td>
</tr>
<tr>
<td>Percutaneous Cystolithotomy</td>
<td>Suitable for any number and size of stones.[125] May allow for more complete removal of stones.[125]</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Different methods for calcium oxalate urocystolith and/or urethrolith removal. Based on Bartges and Polzin, 2011[126]

4. Management and prevention
Unfortunately dogs with CaOx urolithiasis have a high risk of recurrence. One study reported a recurrence rate of 48% within 3 years.[127] In another, Miniature Schnauzers with a history of CaOx cystoliths had a 62% rate of recurrence.[128] Strategies to alter the urine composition combined with ongoing monitoring are recommended. To reduce future formation the American College of Veterinary Internal Medicine consensus recommendations include decreasing urine concentration, reducing calculogenic minerals and avoiding urine acidification. Addressing underlying disease which may contribute to calciuresis and discontinuing medications discussed in Section A are ideal if possible. Monitoring of
urine parameters and screening imaging is also recommended, as early intervention allows for less invasive stone removal methods to be used.

The majority of prevention methods are designed to reduce calciuresis. Various studies have attempted to assess the efficacy of prevention measures, which is often expressed as reduction in RSS and without long term follow up data.

4.1. **Diet composition** Reduction of calculogenic minerals can be achieved by adjusting the composition of the diet. Therapeutic diets which are restricted in calcium and oxalate have been shown to reduce CaOx RSS.[129] Although the recommendation for human calcium oxalate urolithiasis patients is moderate calcium and low oxalate, dogs had the lowest RSS when both calcium and oxalate were reduced. Furthermore, dogs which were fed a therapeutic diet with either moderate or a low calculogenic products (calcium, phosphorus and protein) had less recurrence of calcium oxalate cystic calculi.[130] Lower protein diets were historically recommended to avoid urine acidification associated with purine metabolites, however the effectiveness of this is unproven.

4.2. **Moisture content:** Increasing moisture content of food can increase total water intake in some dogs. Increased water intake will result in a reduced urine specific gravity and RSS. This has been demonstrated in healthy Miniature Schnauzer dogs fed either dry kibble (7% moisture) or soaked in water to achieve 73% moisture content.[131] Even though voluntary water consumption was not accounted for, the soaked-kibble diet was associated with lower USG values, indicating dogs from this group had higher total moisture intake. Another alternative is to increase the sodium content of the food, to promote polydipsia. However, urinary sodium excretion is linked to calciuresis as discussed above. Up to 1.2g/400kcal or 170-230mg/kg/day did not significantly increase urine calcium concentration but did reduce RSS. Therefore, increasing dietary sodium is an alternative for dogs unable to be changed onto a moist diet.[75]

4.3. **pH:** Urine alkalization may be required to achieve a target pH of 7.0. Strategies for this include avoiding excessive dietary protein and the addition of potassium citrate if required. A fasted sample should be used for assessment of pH to avoid recording the transient post-prandial “alkaline tide”.

4.4. **Potassium citrate:** Although supplementary citrate alkalizes urine and decreases the CaOx RSS in humans [94, 132], the result of supplementation on either the pH or CaOx RSS in healthy dogs is less clear.[133] Potassium citrate is sometimes recommended as a therapy based on a study that showed it reduced the RSS in some Miniature Schnauzers.[133] There are several proposed mechanisms why it may have this effect. Firstly, potassium citrate induces a metabolic alkalosis which induces tubular calcium resorption and lowers urinary calcium (see discussion in Section B). Secondly, the metabolic alkalosis increases the urinary citrate levels, which chelates calcium and further reduces urinary calcium (see section B). Thirdly, increased the urinary pH increases the strength of calcium-phosphate binding and activation of other inhibitors such as pyrophosphate (see section B). Despite these theories, human studies fail to consistently find reduced calcium excretion with potassium citrate supplementation. It appears that we currently do not have enough information to make a strong recommendation that potassium citrate will
lower stone risk. Measuring the benefit to the patient (ie reduction in calciuresis and pH after starting therapy) may assist with making individual treatment recommendations.

4.5. **Thiazide diuretics:** In humans, hydrochlorothiazide has been shown to decrease calciuresis[134] and decrease episodes of calcium oxalate urolithiasis.[135] This medication is routinely used to treat humans with idiopathic hypercalciuria. Veterinary research shows dogs with a history of calcium oxalate urolithiasis have decreased urine calcium excretion when treated with hydrochlorthiazide.[136] However research demonstrating reduced urolithaisis recurrence is lacking. Chlorthalidone has also been shown to reduce calculous recurrence in people[137], however this medication is not used routinely in dogs.

Calcium oxalate urolith prevention strategies are largely unproven; supportive veterinary literature is minimal or absent. Most interventions are based on reduction of stone disease in humans or a reduction of RSS. We must extrapolate this to canine patients and assume RSS closely correlates with clinical disease. However as discussed above there are many additional elements apart from RSS that play a part in CaOx formation, including factors which we do not yet understand. In vivo prospective studies are vitally needed to improve our ability to help dogs with this condition.
CHAPTER TWO

SECTION A: INTRODUCTION

Calcium oxalate (CaOx) urolithiasis can be detected in approximately 24% of older, healthy dogs of high-risk breeds such as the Miniature Schnauzer. Recurrence occurs in 48% of cases within 3 years[127] and may be due to a limited understanding of CaOx pathogenesis. Identifying a marker of the disease is the first step to better assess the risk of disease or recurrence.

Urine composition from dogs with CaOx urolithiasis differs from healthy dogs. Relative supersaturation (RSS) [74] and increased urine calcium concentration[74, 105, 106] appear to be the most consistent differences. Of these two, measurement of urinary calcium concentration is more cost-effective and readily available. Although urine calcium excretion (calciuresis) has traditionally been measured by performing 24 hour urine collection, the convenience can be improved by performing a spot measurement. To account for variations in glomerular filtration rates throughout the day, urine calcium concentration can be indexed to concurrent urine creatinine concentration (UCa/Cr). In children, the UCa/Cr correlates moderately well to 24 hour urinary calcium measurements.[138] Therefore, UCa/Cr has the potential to be an attractive alternative for measuring calciuresis in dogs.

Although there is no accepted reference range for the UCa/Cr, a recent study showed a significant difference between fasted control and fasted urolith-forming dogs.[106] Therefore, one of the goals is to assess the UCa/Cr as a diagnostic tool to identify stone status, and determine the most accurate threshold for healthy dogs.

Furthermore, previous data also shows that six urolith-forming dogs had abnormally increased post-prandial calciuresis.[105] A better understanding of variation in calciuresis in regards to fasted and post-prandial states could improve the specificity of using the UCa/Cr measurement to differentiate between healthy and stone-forming dogs. We hypothesized that post-prandial urine calcium excretion kinetics differ between urolith-forming and non-urolith-forming dogs, and these differences could improve the discriminatory power of the UC/Cr.

There are a variety of other patient variables that affect renal excretion of calcium, including sex, breed, diet, concurrent diseases and medications.[109] Any attempt to investigate the effect of postprandial calciuresis should attempt to control for these as much as possible.

Male miniature schnauzers have the highest incidence of CaOx urolithiasis[108], therefore this population was selected for inclusion. We wanted to avoid classifying dogs with CaOx nephroliths as healthy controls, due to the strong association between human CaOx nephrolithiasis and hypercalciuria. Therefore, the inclusion criteria for urolith-former was expanded to include all forms of urolithiasis including nephroliths, urocystoliths or both.

The aims of this study were firstly to quantify differences in fasted and postprandial UCa/Cr between urolith-forming and control Miniature Schnauzers. The second aim was to compare the UCa/Cr in the postprandial period to a fasted baseline in both urolith-forming and control miniature schnauzers. Finally
we investigated if the post-prandial change is significantly different between stone-forming and control dogs.

SECTION B: MATERIALS AND METHODS

Case selection

The study was advertised to clients of the Veterinary Teaching Hospital (VTH) at the Virginia-Maryland College of Veterinary Medicine and to the local community. The proposed experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Tech. Inclusion criteria were adult (> 24 months of age), male (either neutered or intact), Miniature Schnauzer dogs. Dogs with diseases which alter calcium excretion (e.g. hypercalcemia, hyperparathyroidism, renal disease, diabetes mellitus, osteolytic disease, granulomatous disease and hyperadrenocorticism) were excluded. To identify concurrent disease, the medical history was reviewed, in addition to performing a physical exam, biochemistry profile, urinalysis, abdominal radiography and urinary tract ultrasonography. Dogs were excluded if they were not consuming a complete and balanced commercial canine adult maintenance food substantiated per Association of American Feed Control Officials (AAFCO) nutrient profiles or feed test. Specifically, dogs were excluded if they were consuming a prescription urinary diet which would be designed to alter urine constituents. A detailed diet history was collected and reviewed by a board certified nutritionist. Dogs currently receiving medications which are known to alter calcium excretion (glucocorticoids, furosemide, thiazide diuretics, levothyroxine, theophylline or potassium citrate) were also excluded. Dogs were prospectively enrolled with informed owner consent. Therefore, the study population was based on a convenience sample; all dogs that met the inclusion criteria without any of the exclusion factors were enrolled.

Dogs were classified into two groups based on client history, medical records review and results of abdominal radiography and urinary tract ultrasonography (kidney, ureter, bladder) performed by the investigators. The groups were: 1) “Urolith-former” dogs being those with previously removed or currently present calcium oxalate uroliths anywhere in their urinary system, or 2) breed-matched “controls” with no history or current presence of uroliths.

If urolithiasis was identified through either diagnostic imaging or the medical history, the following criteria were then applied to determine if it was reasonable to conclude that uroliths were composed of calcium oxalate. The criteria were either: 1) confirmatory calcium oxalate urolith analysis, or 2) had neutral or acidic urine with lack of evidence of bacterial urinary tract infection, hepatic disease, or crystalluria of any type other than CaOx. Dogs concluded to have other types of uroliths were excluded from the study.

Study procedures

Dogs presented to the VTH the afternoon before sample collection. The Daily Energy Requirement (DER) was calculated using the equation 1.6 X 70 X BW0.75 where BW is the current body weight in kilograms. All dogs were fed a standardized diet to meet the DER, divided into an evening and morning meal, 12 hours apart. Dogs had to eat at least half the prescribed meal, either voluntarily or by syringe feeding. Dogs urinated 3-5 hours after the evening meal, which reduced the likelihood of post-prandial evening urine contributing significantly to the fasted morning sample. The following morning, an initial
urine sample was collected prior to feeding (“fasted baseline”). After the fasted baseline was collected, dogs were fed their morning meal. Urine was collected at four post-prandial time points (referred to as the 1, 2, 4 and 8 hour post-prandial samples).

Urine was collected by free catch whenever possible, but if dogs did not voluntarily urinate, a sample was collected by aseptic urinary catheterization or cystocentesis, whichever was least stressful for individual dogs.

**Laboratory measurements**

Initial fasted blood and urine samples were submitted immediately upon collection to the VITALS Laboratory of the VMVCM for a biochemistry profile and a urinalysis. If the urine dipstick detected 2+ or higher proteinuria, then an SSA bumin test was performed. If this was also positive then a urine protein:creatinine ratio was calculated. If the serum total calcium was outside the laboratory reference range, then an ionized calcium measurement was performed.

The fasted and post-prandial urine samples were submitted immediately for calcium and creatinine measurements. Urine calcium and creatinine concentrations were measured by spectroscopy on all five samples using a Beckman Coulter AU 480 analyser, by the calcium sensitive Arsenazo dye and the modified Jaffe procedures, respectively.

**Statistical analysis**

The primary outcome was UCa/Cr while the primary exposure of interest was urolith status (urolith-former vs control). Several potentially confounding variables including age, body condition, and proteinuria were selected for further analysis. This was based on previously published associations with CaOx formation.[106, 109, 112] Furthermore, because an association between dyslipidemias and proteinuria in Miniature Schnauzers has been described[139], the effects of serum triglycerides and serum cholesterol were also investigated. A initial tentative UCa/Cr reference range for Miniature Schnauzer dogs was extrapolated from previous published literature.[106] This was based on the UCa/Cr interquartile range from miniature schnauzers with no history of urolithiasis, and yielded 0.05 as the upper limit of normal.

To assess for postprandial change, firstly a $\Delta$UCa/Cr was calculated for each dog at each time point using $\text{UCa/Cr}_x - \text{UCa/Cr}_0$, where UCa/Cr$_0$ is the fasted baseline of the individual dog, and $x$ refers to the specific postprandial timepoint. Secondly the % $\Delta$UCa/Cr was calculated using $(\text{$\Delta$UCa/Cr}/\text{UCa/Cr}_0) \times 100$.

Normal probability plots showed that UCa/Cr, $\Delta$UCa/Cr, %$\Delta$UCa/Cr, age, and serum cholesterol levels followed a normal distribution while body condition score and serum triglycerides level were skewed. Normally distributed variables were summarized as means (standard deviation) and skewed variables as medians (range). Categorical variables were summarized as contingency tables. Associations between urolith status and potential confounders were assessed using 2-sample t-tests (age and cholesterol), Wilcoxon rank sum test (body condition score and triglycerides) and Fisher’s exact test (presence of proteinuria, presence of hypercalciuresis). A mixed model ANOVA was used to assess the impact of urolith status (urolith-former vs control) and time (fasted vs 1, 2, 4, 8hr post prandial) on the UCa/Cr, $\Delta$UCa/Cr, and %$\Delta$UCa/Cr (separately). Each model also specified dog identification as a random effect,
and residuals were inspected to verify that the errors were normally distributed with a mean of zero and a constant variance. Age, proteinuria, cholesterol, triglycerides were added to each of the models mixed model ANOVA models but none reached statistical significance (data not shown). Statistical significance was said to be p<0.05. All analyses were performed using SAS 9.4 (Cary, NC, USA).

ROC analysis was performed to determine the most appropriate threshold for using the UCa/Cr to identify CaOx urolithiasis in male Miniature Schnauzers. The mean UCa/Cr for each dog was calculated. In addition, we utilized UCa/Cr data from male miniature schnauzers from a previously published paper [106]. A receiver operator curve was constructed using JMP to determining the ideal UCa/Cr cut-off value with yields maximum sensitivity and specificity for differentiating stone-formers from control dogs.

SECTION C: RESULTS

Twenty-four Miniature Schnauzers were enrolled; nine urolith-formers and fifteen healthy controls. The urolith-formers comprised of two dogs with nephroliths only, one dog with urocystoliths only, and six dogs with nephroliths plus either current or previous urocystoliths. Comparative data for the two groups of dogs are seen in Table 1. Dogs had a mean age of 8 years, with a range from 2-12 years of age. The urolith-formers were older, although this did not reach significance. Dogs were predominately neutered, with the exception of two intact males in the control group. Both groups had a similar body weight and body condition score (mean of 5.6/9). Proteinuria, as detected by SSA Bumin test, was identified in 20% of control dogs and 44% of urolith-formers, although this did not reach statistical significance. Mean protein:creatinine ratio was numerically higher in the urolith formers; however significance was not achieved. Serum cholesterol and triglyceride concentrations were both significantly greater within the urolith-formers compared with the controls.

Across all 120 urine samples collected, the urolith-formers had significantly higher UCa/Cr compared to controls. Furthermore, urolith-formers had greater UCa/Cr, compared to controls, at 1 and 8 hours postprandial (refer to Figure 1). Individual UCa/Cr values are illustrated in Appendix 1.

A significant postprandial effect on UCa/Cr was not appreciated in either group. The ∆UCa/Cr at 1 hour, 2 hours, 4 hours and 8 hours postprandial were not significantly different from zero, and the ∆UCa/Cr was not significantly different between the groups. The %∆UCa/Cr were also not significantly different from zero or between stone-forming and control groups (Figure 2).

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Urolith-formers</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)*</td>
<td>7.2 (± 3.7)</td>
<td>9.4 (±2.0)</td>
<td>0.107</td>
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<tr>
<td>Body Condition Score (9) #</td>
<td>6.0 (±1.0)</td>
<td>5.0 (±1.0)</td>
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<tr>
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<td>98.0 (±147.0)</td>
<td>451.0 (±516.0)</td>
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<td>273.6 (±93.5)</td>
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<td>Presence of proteinuria</td>
<td>3/15</td>
<td>4/9</td>
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Table 1: Variables of control and urolith-former dogs. Normally distributed variables are marked by an asterisk (*); the mean values are listed and the standard deviation in parentheses. Skewed variables are marked by a pound (#) so the median is listed, and the interquartile range in parentheses. Significant p-values are shown in bold font. Hypercalciuresis was initially defined as UCa/Cr >0.05 based on Furrow et al, 2015.[106]

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (Mean ± SD)</th>
<th>Urolith-Former (Mean ± SD)</th>
<th>p-value</th>
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<tr>
<td>UP:C#</td>
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<td>UCa/Cr*</td>
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<tr>
<td>0hr</td>
<td>0.034 (±0.018)</td>
<td>0.052 (±0.031)</td>
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<tr>
<td>1hr</td>
<td>0.031 (±0.017)</td>
<td>0.063 (±0.036)</td>
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<tr>
<td>2hr</td>
<td>0.039 (±0.024)</td>
<td>0.061 (±0.027)</td>
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<td>4hr</td>
<td>0.042 (±0.039)</td>
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<td>8hr</td>
<td>0.044 (±0.017)</td>
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<td>% ΔUCa:Cr*</td>
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<td>8 hours</td>
<td>36.3 (±83.9)</td>
<td>49.4 (±72.7)</td>
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<tr>
<th>Presence of hypercalciuresis (UCa/Cr &gt;0.05)</th>
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Fig 1: UCa/Cr at different postprandial time points in control (white boxes) and urolith-forming (grey boxes) Miniature Schnauzer dogs. The box represents the median and the IQR, the whiskers represent values within
Fig 2: Box plot showing % ΔUCa/Cr for control dogs (white boxes) and urolith-formers (grey boxes). The box represents the median and the IQR. The circles and crosses within boxes represent the mean. The whiskers represent values within 1.5 IQR. Values outside 1.5IQR are considered outliers and represented either circles or crosses outside boxes. There are no significant changes between the groups at any time point.

Based on ROC analysis of these 24 dogs and 34 previously published dogs[106], an optimal UCa/Cr cut-off value of 0.059 (see Appendix 2) was determined. Using this, the UCa/Cr has a sensitivity of 90% and a sensitivity of 64% for detecting CaOx urolithiasis in male Miniature Schnauzers.

SECTION D: DISCUSSION

This study provides additional information on the UCa/Cr measurement in male Miniature Schnauzers, and the association of the UCa/Cr with stone status. It provides further evidence that some Miniature Schnauzers who form CaOx uroliths have abnormal calciuresis. Additionally, we did not observe significant increases in postprandial UCa/Cr, which is contrary to observations by Lulich [105] who
identified postprandial increases in calciuresis in 24-hour collections. Our study does not necessarily exclude differences in postprandial calciuresis, as we used the UCa/Cr as an estimator of calciuresis as previously done by Furrow. Normalizing urine calcium, or any other analyte, to urine creatinine will only lead to a significant linear relationship to 24-hour excretion if both urine calcium and creatinine concentrations remain steady or fluctuate in unison throughout a 24-hour period. Studies in human populations have shown variably significant relationships between spot UCa/Cr and 24-hour urine calcium quantification. Further research into the relationship between these two parameters in dogs is needed. Twenty four hour urine collections would be impractical for the majority of pet owners and veterinarians to collect. The goals of this study were to gain further information on a practical measurement that can be utilized by practitioners to define calciuresis and possibly predict stone risk or status.

However, the UCa/Cr was sensitive enough to detect a significant difference in calciuresis between the affected dogs compared to the control dogs. Although significant differences were identified only at 1 hour and 8 hours postprandially, these specific timepoints are unlikely to reflect a clinically relevant difference from other time points. When examining all individual dog results (see Appendix 1), many affected Miniature Schnauzers have hypercalciuresis (initially defined as UCa/Cr > 0.05) throughout the day. Therefore, if using the UCa/Cr to identify hypercalciuresis, the time of sample collection does not appear to be critical. Furthermore, prolonged urine supersaturation may be one of the mechanisms behind clinical CaOx urolithiasis in Miniature Schnauzers.

If using the UCa/Cr as an indicator of calcium oxalate stone formation, we propose a tentative upper reference interval of 0.06. This maximum UCa/Cr value has an excellent specificity at correctly excluding CaOx urolithiasis in 90% of healthy Miniature Schnauzers. This reference interval has only modest sensitivity; it could be expected to correctly identify 64% of urolith-formers. One reason for the moderate sensitivity of the UCa/Cr is that calcium oxalate urolithiasis in Miniature Schnauzers is presumed to have a multifactorial etiology. Additional factors apart from urinary calcium concentration contribute to CaOx urolith formation. Promoters and inhibitors of urinary crystal initiation, aggregation and growth that are important for development of CaOx urolithiasis in humans likely also contribute to the development of disease in dogs. One example is the urinary macromolecule nephrocalcin, a strong inhibitor of crystalline growth. A defective isoform of this glycoprotein was the predominate phenotype expressed in some stone forming humans and possibly in dogs compared to healthy controls.

The association of CaOx urolithiasis with dyslipidemias and proteinuria was an interesting finding. In this study proteinuria was not identified as a significant factor, which is in contrast to previous findings and likely due to the limited sample size reported here. Humans with Metabolic Syndrome typically have hypercholesterolemia and hypertriglyceridemia, and this is associated with subepithelial tubular damage, development of Randall’s plaques, crystal formation and then nephroliths. It is not known if canine nephroliths form by the same mechanisms, although it is an attractive theory given the association of these co-morbidities. Alternatively, dyslipidemias, proteinuria and calcium oxalate urolithiasis may be common but unrelated conditions in the Miniature Schnauzer.

Though it would not have been appropriate to do with the small population included in this pilot study, with larger studies, regression analysis could be applied to remove confounding variables such as age. Another approach is to use age-matched controls to remove this variable. Unfortunately slow patient
recruitment and low numbers of suitable Miniature Schnauzers prevented age-matched control dog enrollment in this study. Larger studies would also allow regression analysis to identify and quantify contributions from other variables such as proteinuria, hypercholesterolemia and hypertriglyceridemia.

Dietary composition has been previously examined for its role in urolith formation.\textsuperscript{[128]} In this study, comparing the current or previous diets between stone status groups or calciuresis groups could not be done due to the wide variety of diets fed and the lack of specific manufacturer’s nutritional information. We attempted to minimize the effect of diet variation by feeding a standardized diet one meal before and after the fasted sample.

There are several other limitations of this study. First, dogs were given ample opportunity to empty their bladder at each timepoint, but residual urine volumes were not measured. Therefore it is possible that some urine produced during the previous time period may have remained in the bladder and confounded subsequent results.

Secondly, it is possible that some control dogs may develop urolithiasis in the future. The mean age of dogs diagnosed with CaOx urolithiasis is reported to be 8 years,\textsuperscript{[109]} so younger control animals may be ‘latent’ stone formers. It is not known if the UCa/Cr would increase before the onset of clinical disease, although this would be a useful finding to proactively manage high-risk dogs. Ideally control dogs could be followed over time to identify if the UCa/Cr can identify future urolith-formers before the onset of clinically detectable urolithiasis. In addition, following the UCa/Cr of urolith-formers over time could be used to help identify the effectiveness of prevention measures designed to reduce calciuresis. This may be considerably more convenient for investigators than measurement of 24 hour urine calcium excretion, such as performed by Lulich et al to assess the impact of hydrochlorothiazide.\textsuperscript{[136]}

In conclusion, hypercalciuresis is common amongst male urolith-forming Miniature Schnauzers. The UCa/Cr proved to be a simple and cost-effective test that has potential to be used to identify dogs at risk of CaOx urolithiasis. A tentative cut-off for UCa/Cr of male Miniature Schnauzers is 0.06, although further studies will help establish a more widely accepted reference range for normal calcium excretion. If using this, the sensitivity and specificity of the UCa/Cr for CaOx urolithiasis is 64\% and 90\% respectively. Excessive calciuresis appears to occur throughout the day so strict timing of urine collection is likely not required.

\textsuperscript{a} Hills Science Diet Adult 1-6yr Chicken and Barley Entrée Canned Dog Food


Appendix 1. UCa:Cr for control (grey triangles) and urolith-forming (black diamonds) dogs at different timepoints
Appendix 2: Receiver Operator Characteristic

Using Group='Stone-former' to be the positive level

AUC
0.78393

ROC Table

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<th>Sens-(1-Spec)</th>
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<th>True Neg</th>
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