# THE URINARY PROTEOMIC ANALYSIS AND THE VITAMIN D RECEPTOR POLYMORPHISMS IN DOGS WITH CALCIUM OXALATE UROLITHIASIS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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# การวิเคราะห์โปรตีนในปัสสาวะโดยวิธีโปรติโอมิกส์และความหลากหลายของยีนตัวรับวิตามินดีในสุนัขที่เป็นนิ่วชนิดแคลเซียมออกซาเลต

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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สุมนวรรณ แจ่มสุวรรณ : การวิเคราะห์โปรตีนในปัสสาวะโดยวิธีโปรติโอมิกส์และความหลากหลายของยีนตัวรับวิตามินดีในสุนัขที่เป็นนิ่วชนิดแคลเซียมออกซาเลต. ( THE URINARY PROTEOMIC ANALYSIS AND THE VITAMIN D RECEPTOR POLYMORPHISMS IN DOGS WITH CALCIUM OXALATE UROLITHIASIS) อ.ที่ปรึกษาหลัก : ศ. สพ.ญ.ดร.ชลลดา บูรณกาล, อ.ที่ปรึกษาร่วม : รศ. น.สพ.ดร.กฤษ อังคนาพร,รศ. นพ.ดร.ฐสิณัส ดิษยบุตร

นิ่วชนิดแคลเซียมออกซาเลต เป็นนิ่วชนิดหนึ่งที่พบได้บ่อยทั้งในสุนัขและในคน ภาวะระดับแคลเซียมในปัสสาวะที่สูงขึ้น เป็นหนึ่งในสาเหตุโน้มนำของการเกิดโรคนิ่วแคลเซียมออกซาเลตในทางเดินปัสสาวะ กระบวนการเกิดนิ่วชนิดนี้พบว่ามีส่วนเกี่ยวข้องกับปัจจัยทางพันธุกรรมและยังพบความสัมพันธ์ระหว่างระดับแคลเซียมกับความแตกต่างทางพันธุกรรมของตัวรับวิตามินดีในผู้ป่วยที่เป็นนิ่วในหลายกลุ่มประชากร นอกจากนี้โปรตีนที่ตรวจพบในปัสสาวะบางชนิดก็มีส่วนเกี่ยวข้องกับกระบวนการเกิดนิ่วเช่นกัน การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ของความแตกต่างทางพันธุกรรมของตัวรับวิตามินดีกับการเกิดนิ่วชนิดแคลเซียมออกซาเลตในสุนัข และเพื่อศึกษาการแสดงออกของโปรตีนในปัสสาวะของสุนัขที่เป็นนิ่วชนิดแคลเซียมออกซาเลตและมีภาวะแคลเซียมสูงในปัสสาวะ ในการนี้จึงแบ่งการศึกษาออกเป็น 2 ส่วน การศึกษาในส่วนแรกทำการแบ่งสุนัขออกเป็น 2 กลุ่ม ได้แก่ กลุ่มควบคุม จำนวน 40 ตัว และกลุ่มที่เป็นนิ่วชนิดแคลเซียมออกซาเลต จำนวน 35 ตัวการเก็บตัวอย่างเลือดเพื่อตรวจวิเคราะห์ค่าทางโลหิตวิทยา ระดับอิเล็กโทรไลต์ ระดับวิตามินดี และเพื่อตรวจวิเคราะห์รหัสพันธุกรรมของยีนตัวรับวิตามินดี ตำแหน่ง rs852900542 และ rs851998024 ทำการตรวจวิเคราะห์ปัสสาวะเพื่อหาความเข้มข้นของอิเล็กโทรไลต์ ในการศึกษาส่วนที่ 2 ศึกษาชนิดและความแตกต่างของโปรตีนในปัสสาวะของสุนัขที่เป็นนิ่วและมีภาวะแคลเซียมในปัสสาวะสูง จำนวน 7 ตัว เปรียบเทียบกับสุนัขกลุ่มควบคุมที่มีสายพันธุ์ เพศ และอายุเดียวกัน จำนวน 7 ตัว ผลของการศึกษาในส่วนที่ 1 พบว่า มีความแตกต่างของสัดส่วนจีโนไทป์ของยีนวิตามินดี ตำแหน่ง rs852900542 ระหว่างกลุ่มสุนัขที่เป็นนิ่วและกลุ่มสุนัขควบคุม ($P<0.05$) และยังพบว่าสุนัขที่มีจีโนไทป์แบบ CT หรือ CC มีความเสี่ยงในการเกิดนิ่วแคลเซียมออกซาเลตสูงกว่าสุนัขที่มีจีโนไทป์แบบ TT (OR = 3.82, 95% CI 1.04 – 13.98, $P<0.05$). นอกจากนี้สุนัขที่เป็นนิ่วที่มีจีโนไทป์แบบ CT หรือ CC จะมีระดับแคลเซียมและแมกนีเซียมในปัสสาวะที่สูงกว่าสุนัขเป็นนิ่วที่มีจีโนไทป์แบบ TT ($P<0.05$) อย่างไรก็ตามไม่มีความแตกต่างของยีนวิตามินดีที่ตำแหน่ง rs851998024 ระหว่างสุนัขที่เป็นนิ่วและกลุ่มควบคุม ในส่วนของการศึกษาส่วนที่ 2 สามารถตรวจวิเคราะห์โปรตีนได้ทั้งหมด 49 ชนิดที่พบได้ทั้งสุนัขที่เป็นนิ่วและกลุ่มควบคุมที่มีภาวะแคลเซียมสูงในปัสสาวะ โดยพบว่าโปรตีน thrombomodulin มีระดับสูงกว่าอย่างมีนัยสำคัญในกลุ่มสุนัขที่เป็นนิ่ว เมื่อเปรียบเทียบกับสุนัขกลุ่มควบคุม ($P<0.05$) นอกจากนี้ระดับของโปรตีน VIP36 และ pantetheinase ในปัสสาวะของกลุ่มสุนัขที่เป็นนิ่วเพิ่มขึ้น ($P=0.16$ and $P=0.17$ ตามลำดับ) ในขณะที่โปรตีน ICAM-1 มีปริมาณลดน้อยลง เมื่อเปรียบเทียบกับสุนัขกลุ่มควบคุม ($P=0.14$) โดยสรุปได้ว่า ความแตกต่างทางพันธุกรรมของยีนตัวรับวิตามินดี ตำแหน่ง rs852900542 มีความเกี่ยวข้องต่อความไวในการเกิดนิ่วชนิดแคลเซียมออกซาเลตในสุนัข โดยสัมพันธ์กับการขับทิ้งแคลเซียมในปัสสาวะ นอกจากนี้การเพิ่มขึ้นของโปรตีนในปัสสาวะในสุนัขที่เป็นนิ่วและมีภาวะแคลเซียมในปัสสาวะสูง ได้แก่ โปรตีน thrombomodulin pantetheinase และ VIP36 ซึ่งอาจเป็นประโยชน์ในการบ่งชี้ถึงภาวะการบาดเจ็บในทางเดินปัสสาวะ ข้อมูลจากการศึกษาทางพันธุกรรมนี้อาจเป็นประโยชน์ในการคัดกรองความเสี่ยงและป้องกันการเกิดนิ่วแคลเซียมออกซาเลต และการพบโปรตีนในปัสสาวะที่อาจใช้เป็นตัวบ่งชี้การบาดเจ็บในทางเดินปัสสาวะในสุนัขที่เป็นนิ่วชนิดแคลเซียมออกซาเลต

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| สาขาวิชา | สรีรวิทยาการสัตว์ | ลายมือชื่อนิสิต ..... |
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Sumonwan Chamsuwan : THE URINARY PROTEOMIC ANALYSIS AND THE VITAMIN D RECEPTOR POLYMORPHISMS IN DOGS WITH CALCIUM OXALATE UROLITHIASIS. Advisor: Prof. Dr. CHOLLADA BURANAKARL Co-advisor: Assoc. Prof. Dr. KRIS ANGKANAPORN,Assoc. Prof. Dr. THASINAS DISSAYABUTRA

Calcium oxalate (CaOx) urolithiasis is one of the most common stone components which frequently occurs in both humans and dogs. Hypercalciuria is one of the predisposing factors commonly found in both people and dogs with calcium urolithiasis. The genetic factors are also involved with the pathogenesis of stone formation, and the relationship between calcium handling and vitamin D receptor (VDR) polymorphisms has been demonstrated to be related to calcium urolithiasis in human populations. Moreover, some urinary proteins may be involved in the process of stone formation. The present study aimed firstly to evaluate the relationship between *VDR* polymorphism in dog with CaOx urolithiasis. Secondly, the urinary proteomic profile between hypercalciuric dogs with or without CaOx urolithiasis was investigated. The study was divided into two parts: part I and part II. The study in part I was divided into 2 groups, CaOx dogs (n=35) and stone-free control dogs (n=40). The blood sample was collected for determination of complete blood count, serum chemistry profiles, serum electrolytes, serum vitamin D, and DNA analysis for single nucleotide polymorphism (rs852900542 and rs851998024) of the *VDR* gene. The urine sample was collected for determination of electrolyte concentrations. In study part II, the urinary proteomic profiles were determined in CaOx stone dogs with hypercalciuria (n=7) compared with the breed, age-, and sex-matched hypercalciuric controls (n=7). The results from the study in part I showed that the genotypic distribution of rs852900542 was significantly different between CaOx stone and control dogs ($P<0.05$), and dogs with a CC or CT genotypes had an increased risk for CaOx stones than those with the TT genotype (OR = 3.82, 95% CI 1.04 – 13.98, $P<0.05$). Moreover, CaOx dogs with CC or CT had higher UCa/Cr and UMg/Cr than those with the TT ($P<0.05$). However, there was no difference in genotypic distribution of rs851998024 between CaOx dogs and control dogs in this study. In the study part II, 49 proteins were identified in urine from both hypercalciuric CaOx stone former and stone-free dogs. Thrombomodulin was significantly higher between the control and case groups ($P<0.05$). The vesicular integral-membrane protein (VIP) 36 and pantetheinase were higher in CaOx stone-former ($P=0.16$ and $P=0.17$, respectively), while intercellular adhesion molecule 1 was reduced ($P=0.14$). In conclusion, the rs852900542 VDR polymorphism is associated with CaOx susceptibility in dogs, which is related to urinary calcium excretion. In addition, the hypercalciuric CaOx dogs have an increased level of urinary proteins, including thrombomodulin, pantetheinase ,and VIP36 which indicate the urinary tract injury. This genetic finding might be useful for screening dogs that are at increased risk and prevention of CaOx urolithiasis, and the identification of urinary proteins may be useful for the urinary tract injury marker in dogs with CaOx urolithiasis.



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| Field of Study: | Animal Physiology | Student's Signature ..... |
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Sumonwan Chamsuwan

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| | |
|---|---|
| 1,25(OH)2D | 1,25-dihydroxyvitamin D3 |
| 24,25(OH)2D3 | 24, 25-dihydroxyvitamin D3 |
| 25(OH)D3 | 25-hydroxyvitamin D3 |
| ACN | Acetonitrile |
| ALB | Albumin |
| ALP | Alkaline phosphatase |
| ALT | Alanine aminotransferase |
| APS | Ammonium Persulfate |
| BLAST | Basic Local Alignment Search Tool |
| BSA | Bovine serum albumin |
| BUN | Blood urea nitrogen |
| Ca | Calcium |
| $Ca^{2+}$ | Ionized calcium |
| CaOx | Calcium oxalate |
| CaP | Calcium phosphate |

| | |
|---|---|
| CBC | Complete blood count |
| CI | Confidence interval |
| Cl- | Chloride |
| Cr | Creatinine |
| CS | Chondroitin sulfate |
| DBP | Vitamin D binding protein |
| DTT | Dithiothreitol |
| DW | Distilled water |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| F | Intact female |
| FA | Formic acid |
| Fs | Spayed female |
| GAGs | Glycosaminoglycans |
| GHS | Genetic hypercalciuric stone-forming rat |
| HA | Hyaluronic acid |
| IAA | Iodoacetamide |

| | |
|---|---|
| ICP-OES | Inductively coupled plasma optical emission spectrometry |
| IH | Idiopathic hypercalciuria |
| LB | Luria-Bertani |
| LBD | Ligand-binding domain |
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| M | Intact male |
| Mc | Castrated male |
| Mg | Magnesium |
| NaOAca | Sodium acetate |
| NC | Nephrocalcin |
| OPN | Osteopontin |
| OR | Odds ratio |
| P | Phosphorus/phosphate |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffer solution |
| PCR | Polymerase Chain Reaction |
| PTH | Parathyroid hormone |

| | |
|---|---|
| RXR | Retinoid X receptor |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SNP | Single nucleotide polymorphism |
| TEAB | Triethylammonium bicarbonate |
| TFF1 | Trefoil factor 1 |
| THP | Tamm-Horsfall protein |
| TMB | Tetramethylbenzidine |
| TP | Total protein |
| UCa/Cr | Urinary calcium to creatinine ratio |
| UMg/Cr | Urinary magnesium to creatinine ratio |
| UP/Cr | Urinary phosphate to creatinine ratio |
| UPC | Urine protein to creatinine |
| UPTF-1 | Urinary prothrombin fragment-1 |
| UTR | 3’ untranslated region |
| VDR | Vitamin D receptor |
| VDRE | Vitamin D responsive element |

# CHAPTER I

# INTRODUCTION

## Background and rationale

Calcium oxalate (CaOx) is one of the most common types of uroliths in dogs, and the prevalence of canine CaOx urolithiasis has increased in most countries since the early to mid-2000s (Lulich et al., 2013). Either genetic or environmental factors might involve in the pathogenesis of CaOx stone formation. Despite multiple underlying causes, most cases of CaOx urolithiasis in humans are idiopathic, and patients could have one or more metabolic risk factors such as hypercalciuria, hyperoxaluria ,and hypocitraturia, which promote calcium stone formation (Kohjimoto et al., 2013). It is well established that idiopathic hypercalciuria (IH) is related to normal serum calcium level and low urinary calcium reabsorption, which are mainly controlled by three major regulating hormones including parathyroid hormone (PTH), calcitonin ,and 1,25-dihydroxyvitamin D3 (1,25(OH)2D) (Worcester and Coe, 2008). In dogs, neither hyperoxaluria nor hypocitraturia seemed to be a risk factor in urolithiasis (Lulich et al., 1991; Furrow et al., 2015). CaOx stone-forming dogs had an abnormal high urinary calcium concentration (Furrow et al., 2015), while the excretion of phosphate was slightly reduced similar to humans (Stevenson et al., 2003). These effects could be mediated by the action of 1,25(OH)2D.

The 1,25(OH)2D or calcitriol, the active form of vitamin D, and its specific intracellular receptor, called the vitamin D receptor (VDR), play a vital role in calcium and phosphorus homeostasis. Calcitriol binds with VDR to exert bone resorption, intestinal absorption, and renal reabsorption of calcium. A study in patients with IH showed the increase of VDR level in peripheral blood monocytes, although serum vitamin D level was within normal level (Favus et al., 2004). An increased effect of vitamin D in target tissues could result from an increased level of VDR (Favus et al., 2004). Furthermore, the overexpression (or over-activity) of VDR is involved in stone formation in genetic hypercalciuric stone-forming (GHS) rats (Tsuruoka et al., 1997). Interestingly, previous studies reported that GHS rats and some groups of patients with IH failed to reduce urinary calcium excretion by dietary calcium restriction, suggesting a defect in renal calcium tubular reabsorption (Coe et al., 1982; Frick et al., 2013)



Recently, the relationship between calcium handling and VDR polymorphisms has been widely explored. The allelic variations in *VDR* genes including *FokI, BsmI, ApaI, TaqI* ,and *Tru9I* have been demonstrated to be associated with calcium urolithiasis in some populations (Bid et al., 2005; Goknar et al., 2016). Thus, the single nucleotide polymorphisms (SNPs) of VDR may play a crucial role in stone-forming patients. In dogs, multiple *VDR* genes polymorphisms have been reported. However,

the effect of VDR polymorphisms on calcium metabolism and risk for CaOx urolithiasis in dogs is unknown.

To prevent the recurrence of urolithiasis, many investigators pay attention to the pathophysiological of kidney stone formation and proteomic approaches. Basically, CaOx stone induces an inflammatory process to the cells which subsequently leads to epithelial injury or even cellular death. Moreover, this process may produce several proteins which stimulate or inhibit the formation of stone which are considered as candidate biomarkers for CaOx kidney stone formation (Kaneko et al., 2012; Okumura et al., 2013; Boonla et al., 2014). However, there are limited reported data of urinary marker for CaOx urolithiasis in dogs.

**Objectives of Study**

1) To investigate the association between VDR polymorphisms and risk of CaOx urolithiasis in dogs.



2) To investigate the candidate urinary biomarker in hypercalciuric dogs with CaOx urolithiasis dogs using proteomic study and bioinformatic analysis.

**Keywords (Thai):** นิ่วแคลเซียมออกซาเลต, สุนัข, สนิป, ตัวบ่งชี้ทางชีวภาพในปัสสาวะ, ตัวรับวิตามินดี

**Keywords (English):** calcium oxalate stone, dog, SNP, urinary biomarker, vitamin D receptor

**Research hypothesis**

1. The polymorphisms of *VDR* gene are associated with CaOx stone formation in dogs.

2. There are different proteins between urine proteomic profiles of hypercalciuric CaOx urolithiasis compared with hypercalciuric stone-free dogs.

# CHAPTER II

# LITERATURE REVIEW

## A. Urolithiasis in human

Urolithiasis (or stone disease in the urinary tract) is one of the most important urological diseases in human, and its prevalence is increasing across the world (Romero et al., 2010). Approximately 80% of kidney stones are primarily composed of CaOx mixed with calcium phosphate (CaP) (Khan et al., 2016). Factors that increase the risk of urolithiasis development include obesity, diabetes ,and metabolic diseases (Taylor et al., 2005). Genetic factors are also involved in the pathogenesis of stone formation. In addition, the formation of kidney stones is associated with an increased risk of hypertension (Cappuccio et al., 1990), metabolic bone disease (Sakhaee et al., 2011), cardiovascular disease (Ferraro et al., 2013), and chronic kidney disease (Saucier et al., 2010). The most common metabolic syndrome is idiopathic hypercalciuria. At least 30% of the first-time stone formers will have one or more recurrences within 10 years (Singh et al., 2015). Moreover, the high recurrent rate was associated with a shorter interval between each episode (Strauss et al., 1982). Several surgical approaches have been invented to minimize invasive procedures. Furthermore, conventional medical treatments such as dietary sodium restriction, adequate fluid intake, potassium citrate supplement ,and thiazide diuretics are

practical and effective methods for stone prevention (Finkielstein and Goldfarb, 2006).

**B. Urolithiasis in dogs**

In dogs, urolithiasis is a common disease in dogs with frequent recurrence. Most of urinary stones occur in lower urinary tracts such as urinary bladder and urethra which are different from human (Houston et al., 2017; Hunprasit et al., 2017). However, much research has been focused on urinary stone formation in dogs because they spontaneously form stones that physically and chemically resemble those found in human. Urolithiasis accounts for 0.4 – 2% of dogs presented to veterinary hospitals (Bovee and McGuire, 1984). The Minnesota Urolith Centre reported the incidences of canine urolith submissions have increased by 2.5 times over the past decade (Lulich et al., 2013). In many geographic locations, CaOx is the second predominant component of canine uroliths next to struvite, whereas the proportion of CaOx has increased during the last decade (Wisener et al., 2010) (Hunprasit et al., 2017). In Thailand, likewise, the occurrence of CaOx submission significantly increased from 21.2% in 2009 to 32.4% in 2015 (Hunprasit et al., 2017). Risk factors for the incidence of canine CaOx are males, middle-aged to older, and small-breed dogs. The prevention and treatment of canine urolithiasis are similar to human such as laser lithotripsy, surgical removal ,and dietary modification.

**C. Role of calcium and vitamin D metabolism**

1) Calcium metabolism

Calcium is an essential mineral for many physiological processes in the body, including nerve transmission, muscle contraction, coagulation, and bone mineralization. Calcium also works as an intracellular secondary messenger in cell signaling pathways that regulate cell growth and cell proliferation. Normally, 99% of total calcium in the body is stored in the bones and teeth, and only 1% is found in body fluid. The calcium in plasma exists in three forms; approximately 50-60% is free ionized form ($Ca^{2+}$), 40% is bound to plasma proteins which mostly albumin, and 10% complexes with other anions, such as citrate and phosphate (Robertson and Marshall, 1979). Calcium homeostasis is regulated at three major organs: gut, kidney, and bone, through the factors responsible for hormonal and enzymatic activation of PTH, calcitonin ,and 1,25(OH)2D (Peacock, 2010).



2) Abnormal urinary excretion of calcium in calcium urolithiasis

Hypercalciuria is one of the predisposing factors commonly found in calcium urolithiasis patients (Worcester and Coe, 2008). An increase in urinary calcium excretion is directly related to the elevation of urine supersaturation which favors the formation of calcium stones. Although many underlying causes such as primary hyperparathyroidism, hyperthyroidism, excessive vitamin D, renal tubular acidosis, sarcoidosis, or malignancies are associated with hypercalciuria, most hypercalciuric

calcium stone formers have no systemic illness (Park and Pearle, 2007). The hypercalciuric condition with normal serum calcium level is also described as IH. The hypercalciuric nephrolithiasis may be inherited and accounted for 35-65% of patients (Stechman et al., 2009), and IH could be either monogenic or polygenic disorder (Lerolle et al., 2002; Gamboro et al., 2004). Patients with IH might have been classified by different pathogenic mechanisms including increased gut calcium absorption, decreased renal calcium resorption ,and increased bone resorption (Coe et al., 2016).

In dogs, many studies reported abnormal high urinary calcium excretion in dogs with CaOx urolithiasis compared with controls (Stevenson et al., 2003; Dijcker et al., 2012; Furrow et al., 2003; Furrow et al., 2017). Both intrinsic and environmental factors such as lifestyle and living environment may influence the pathophysiology of hypercalciuria in dogs (Lekcharoensuk et al., 2000), although the relationships between these factors and CaOx stone are still unclear.



3) Vitamin D metabolism

Vitamin D is produced from 7-dehydrocholesterol to provitamin D3 and cholecalciferol in the skin through the ultraviolet exposure in human. Nevertheless, dogs and cats insufficiently synthesize cholecalciferol due to low 7-dehydrocholesterol accumulation in the skin (How et al., 1994). The dietary sources such as cod liver oil, dairy products, egg and fish, can naturally provide two main

forms of vitamin D, including ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). Cholecalciferol binds to the specific vitamin D binding protein (DBP) in bloodstream and transports to the liver where 25-hydroxyvitamin D3 (25(OH)D3) is produced by cytochrome P-450 vitamin D-25 hydroxylase (encoded by CYP27A1). This product is transported to the renal proximal tubule and metabolized by 1α-hydroxylase (encoded by CYP24B1 gene) to 1,25(OH)2D or calcitriol, an active form of vitamin D (Deeb et al., 2007). Additionally, the kidney can also synthesize the inactive metabolite or 24, 25-dihydroxyvitamin D3 (24,25(OH)2D3) by 24α-hydroxylase (encoded by CYP24A1) which is excreted into urine. The 1,25(OH)2D can regulate the synthesis of CYP24A1, leading to an increase 24,25(OH)2D3 and 1,24,25(OH)3D3 production. Similarly, 24,25(OH)2D3 can also stimulate the synthesis of 1,25(OH)2D. (Figure 1)

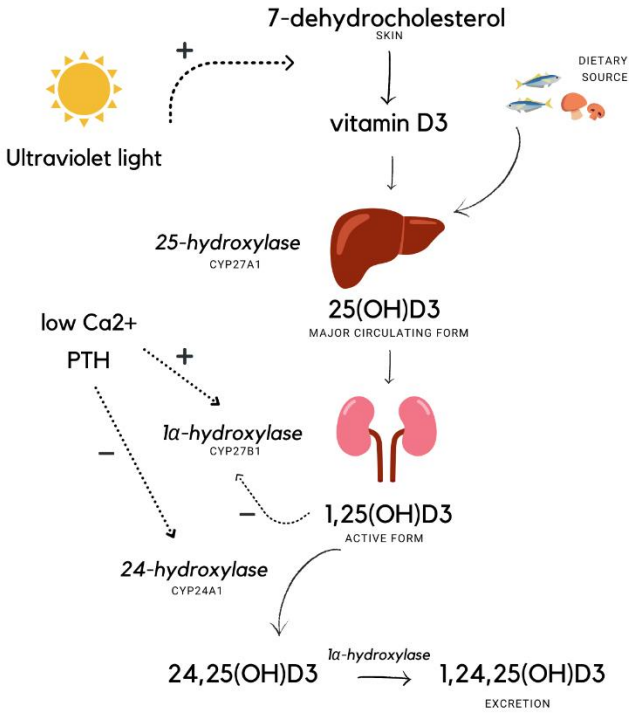


**Figure 1.** Pathway of vitamin D metabolism

4) Vitamin D receptor

The 1,25(OH)2D and its metabolites bind to their specific intracellular receptor, called the vitamin D receptor (VDR). VDR is the member of the nuclear receptor superfamily, activates or represses gene expression in the target cells. VDR structure consists of three domains, the N-terminal DNA-binding domain (DBD), the C-terminal ligand-binding domain (LBD), and the short region binding these two domains together (Mangelsdorf et al., 1995). The DBD of the VDR recognizes the specific DNA sequence known as vitamin D responsive elements (VDREs). Recently, there are many different types of VDREs, called coregulators, that interact with the

VDR to activate or inhibit the transcriptional activity (Bikle, 2000). In addition, the LBD plays a crucial role in heterodimerization with the retinoid X receptor (RXR), which leads to the physiological response. The VDR interacts with VDREs within the promotor of vitamin D responsive gene, leading to transactivation of target genes that control calcium and phosphorus homeostasis, calls classical signaling pathway (Jones et al., 1998). On the other hand, the non-classical signaling pathway is responsible for cell proliferation and differentiation, hormone secretion ,and immune modulation (Bikle, 2000). The 1,25(OH)2D and VDR complex also exerts the non-genomic action via activation of signaling molecules (such as phospholipase C or phospholipase $A_2$) or secondary messenger pathways including $Ca^{2+}$, cyclic AMP, protein kinase A, and protein kinase C which possible leads to the opening of calcium ($Ca^{2+}$) or chloride ($Cl^-$) channels (Norman, 2006) (Figure 2).

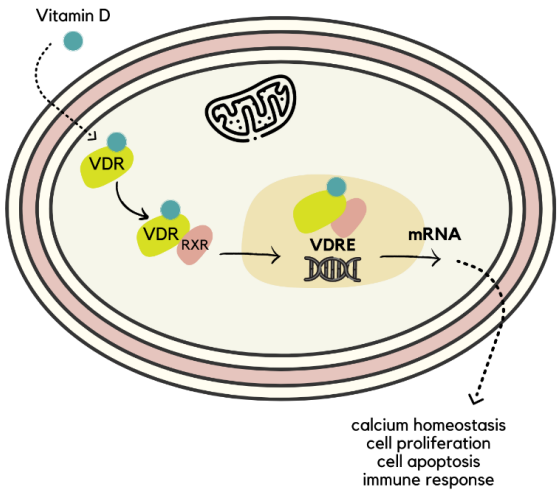


**Figure 2.** Intracellular signaling pathway of vitamin D (1,25(OH)2D)

**D. Single nucleotide polymorphisms of *VDR* gene**

Due to the crucial role of vitamin D in calcium homeostasis, the genetic alterations of the *VDR* gene could affect vitamin D action such as renal calcium handling, leading to stone formation. Recently, there are several epidemiologic evidence of allelic variation in the *VDR* gene that may involve in calcium stone etiology in some human populations. The most common SNPs in *VDR* genes are *ApaI, BsmI, TaqI* ,and *FokI*. The *ApaI, BsmI* ,and *TaqI* which located in the 3' untranslated region (UTR) can affect the mRNA stability and protein translation efficiency (Morrison et al., 1994). While *FokI* locating at 5' end (exon2) of the *VDR* gene relates to transactivation of the vitamin D (Gross et al., 1998). However, the relationship between the observed polymorphisms and urolithiasis susceptibility is still debated. For instance, Ruggiero and colleagues (1999) found that patients with bb haplotype of the *BsmI* polymorphism represented in higher daily urinary calcium excretion. The frequency of the AA *ApaI* genotype was higher in hypercalciuric stone-forming children in Turkey, and the TT *TaqI* genotype was associated with the strength of positive family history (Ozkaya et al., 2003). Another study in pediatric Turkish patients reported that the B allele of *BsmI* and the A allele of *ApaI* was predominant in both hypercalciuric stone formers and normocalciuric stone formers (Goknar et al., 2016). There is also a report that demonstrated the association between the C/T *FokI* genotype and stone formation (Bid et al., 2005). The *TaqI* t allele was found to be associated not only with urinary calcium excretion but also

the severity of stone disease (Nishijima et al., 2002). However, there was not statistically significant of *ApaI, FokI* ,and *TaqI* polymorphisms between the stone formers and healthy controls in Korean study (Seo et al., 2010). Further investigations are needed to understand the genetic factors affecting the pathogenesis of stone formation and to find the suitable candidate gene marker for urolithiasis susceptibility.

Regarding the genetic variation of the *VDR* gene, multiple *VDR* gene polymorphisms have been reported in dogs and are listed in Ensembl (http://www.ensembl.org/), a database of vertebrate genomes and genetic variation. The rs851998024, a missense variant and the only non-synonymous variant in canine VDR, and rs852900542, which is located at the intron region of the *VDR* gene were previously studied (Rodriguez-Cortes et al., 2017). However, the effect of VDR polymorphisms on calcium metabolism and risk for CaOx urolithiasis in dogs is unknown.



**E. Mechanism of urinary stone formation**

1) Mechanism of crystal formation

Normally, the processes involved in the formation of urinary stones are composed of crystal nucleation, growth, aggregation, and retention. Urine supersaturation, a crucial process for urolith formation in various stone-forming minerals, is required for the first step of crystallization or crystal nucleation. However,

normal urine can also be supersaturated without stone formation. Therefore, the urinary constituents are suggested to play a determinant role in urinary crystallization, especially on calcium oxalate, by the balance between urinary-promoting and inhibiting factors (Rimer et al., 2017). In addition, the stone-forming processes will also depend on the nucleation inhibitors and the length of time of incubation (Robertson, 1973). Once crystal nucleation occurs, the establishment of the stone nucleus can be activated by either free particle or fixed particle pathways (Khan et al., 2016). The renal epithelial cells which expose to crystals, produce the macromolecules that become coated organic matrices and lead to stone growth. The rate of crystal growth mainly depends on the supersaturation level and some modifiers including magnesium, citrate, glycosaminoglycans (GAGs), α-1-microglobulin, osteopontin (OPN), Tamm-Horsfall protein (THP), calgranulin, and urinary prothrombin fragment-1 (UPTF-1) (Khan et al., 2016).



2) The stone components

About 95% of the stone matrix is composed of crystalline components, while 5% are organic components that compose of different types of proteins (Khan and Kok, 2004). The most frequent crystalline substances are CaOx, CaP, magnesium ammonium phosphate (or struvite), and purine derivatives such as uric acid. Moreover, most urinary stones contain more than one crystalline components which

are termed multicomponent stones, particularly, CaOx as monohydrate and dihydrate (Boyce, 1968).

The stone matrix also contains proteins, glycosaminoglycans (GAGs), carbohydrates ,and lipids. Most stone matrix proteins have been identified. The first discovery of proteins in human stone matrix were albumin, THP, α ,and γ- globulin (Boyce and Garvey, 1956). The other proteins are also detected in stones, for example, OPN, nephrocalcin (NC), inter-α- inhibitor, α-1-microglobulin, UPTF-1, and calgranulin (Atmani and Khan, 2002; Aggarwal et al., 2013). Some of these proteins have been demonstrated to influence the crystallization of CaOx. Furthermore, hyaluronic acid (HA) and heparin sulfate (HS) are the two main types of GAGs presented in the CaOx stone matrix and urine, whereas chondroitin sulfate (CS) in the absence of HS is the most abundant GAGs found in magnesium ammonium phosphate calculi (Nishio et al., 1985). These results suggested the selective incorporation of GAGs into different types of stones. In addition, some components of the stone matrix are commonly found in the urine. Most of these proteins can be detected using proteomic analysis.



**F. Urinary proteomes in calcium urolithiasis**

Although urolithiasis is generally a consequence of urine supersaturation, most people with supersaturated urine did not develop kidney stone. Thus, urinary macromolecules could involve in the formation of stone. Up to date, many kidney

stone matrix proteins have been widely investigated. Because urine could be a primary source of kidney stone matrix formation, most studies have been focused on the role of urinary constituents in stone pathogenesis. Chutipongtanate and colleagues (2005) demonstrated a urinary stone inhibitor, trefoil factor 1 (TFF1), which its concentration and relative amount were significantly lower in calcium stone-formers compared with healthy control subjects. A study using two-dimensional electrophoresis and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis demonstrated that urinary uromodulin was diminished in the first-time stone-formers compared to the recurrent renal calculi and healthy subjects (Wai-Hoe et al., 2009), suggesting that uromodulin was a candidate biomarker only in first time stone-forming patients.

Furthermore, the quantitative proteomic comparison from both pooled and individual urines from urolithiasis patients and healthy controls showed that ceruloplasmin, an acute-phase protein, was significantly higher in stone-formers (Wright et al., 2011). A recent study discovered that the proteins involved in inflammation and fibrosis, including S100A8 or calgranulin A and fibronectin can be frequently detected in the stone matrix and urolithiasis urine from the same patients (Boonla et al., 2014).

It has been demonstrated that the negatively charged proteins such as OPN, albumin, THP, bikunin, inhibit one or more crystallization processes in vitro. According

to the study of Kolbach-Mandel and colleagues (2017), the protein distribution in urines from stone formers showed increased content of polycations compared to normal subjects. However, each macromolecule acting as an inhibitor or promoter which might be related to post-translation modification was previously mentioned (Kleinman et al., 2004; Viswanathan et al., 2011).

The proteomic studies in Veterinary Medicine have been extensively interested over the last decade. The previous comprehensive proteomic analysis of canine urine showed that many proteins have been similarly identified as in human urine(Brandt et al., 2014). A few studies have been investigated the urinary and stone matrix proteins in stone-forming dogs. The urinary excretion of both THP and GAGs was lower in stone-forming Dalmatians than controls (Carvalho et al., 2003). In contrast, the THP could not be identified in both stone matrix and urine in dogs among different types of urinary stones (struvite, CaOx ,and uric acid) (Forterre et al., 2006).

# CHAPTER III

# MATERIALS AND METHODS

## 1. Study Population

Seventy-five dogs at the Small Animal Hospital, Chulalongkorn University were included in this study. Informed consent was obtained from the owners between February 2019 and January 2020. The dogs in the control group (n=40) had no history of uroliths or lower urinary tract diseases as confirmed by radiography or ultrasonography. The case group (n=35) were previously diagnosed with CaOx stone disease during any episode and were treated at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University. The stone composition was analyzed by standard stone analysis (polarizing light microscopy and infrared spectroscopy) at the Minnesota Urolith Center, USA ,and the major composition of uroliths was CaOx (≥ 70 %). Dogs were excluded from the study if they had remnant stones detected by either abdominal radiographs or ultrasonography or had active urinary tract infection. Dogs that received any kind of drugs that alter urinary calcium excretion (such as glucocorticoid, furosemide, thiazide diuretic, potassium citrate, calcium, and vitamin D supplementation) or were affected by any diseases that change calcium excretion, were also excluded.

The control dogs consumed a regular adult diet that was commercially available, while dogs included in the stone-forming cases (n=35) consumed a

prescription diet for controlling urolithiasis (Canine urinary S/O, Royal Canin Veterinary Diet, Waltham Centre for Pet Nutrition, USA or c/d Multicare Canine Prescription Diet, Hill's Pet Nutrition Inc., USA).

**2. Experimental Protocol**

All procedures were approved by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Faculty of Veterinary Science, Chulalongkorn University (Protocol number 1831101). Informed consent was obtained from the owners of each participant in the study.

Blood and urine samples from case groups were collected at least 14 days after stone removal. The control samples were obtained from the dogs that presented for health checkups or neuter appointments. Before the experimental study, all dogs were subjected to fasting for at least eight hours with free access to water. The general information including sex, age, breed and dietary information was recorded during physical examination. On the experimental day, approximately 5 ml of blood samples were collected via cephalic or saphenous vein into tubes containing ethylenediaminetetraacetic acid (EDTA) (3 ml) for complete blood count (CBC) and DNA extraction. Another portion of the blood (2 ml) was collected in a heparin tube. The plasma was separated and stored at -20 °C for analysis of blood chemistry profiles (alanine aminotransferase; ALT, alkaline phosphatase; ALP, blood

urea nitrogen; BUN, creatinine; Cr, total protein; TP, albumin; ALB), electrolyte concentrations (Ca, P, and Mg), and 1,25(OH)2D levels.

Approximately 10 ml of urine samples were collected via voiding or catheterization. Urinalysis was performed within 1 hour after collection. The urine samples were stored at -80 ℃ for further analysis of electrolytes (Ca, P, and Mg), and creatinine levels.

**3. Analytical Procedures**

**3.1 Study part I**

**3.1.1 Sample preparation for genotyping of VDR polymorphisms**

**3.1.1.1 DNA collection and extraction**

Peripheral blood mononuclear cells (PBMCs) were separated from the blood EDTA-samples using the Ficoll-Paque method (Fuss et al., 2009). The DNA from PBMC was isolated using phenol-chloroform-isoamyl alcohol extraction technique as described previously (Fan and Gulley, 2001). In brief, 400 µl of lysis buffer (10 mM Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0 ,and 0.5% SDS) was added to 100 µl of PBMC sample, then the 10 µl of proteinase K (20 mg/ml) was mixed in a microcentrifuge tube. The mixture was incubated at 50 °C for one hour. The 250 µl of phenol and 250 µl of chloroform : isoamyl alcohol (49 : 1) were added to the mixture, and vortexed thoroughly at 14,000 rpm, in 4 °C for 30 minutes. Then, the upper aqueous phase was transferred to a new microcentrifuge tube. The 4 µl of glycogen (20

mg/ml) with 800 µl of cold absolute ethanol and 400 µl of 2M sodium acetate (NaOAca) were added and subsequently incubated at -70 °C for 30 minutes and centrifuged at 14,000 rpm in 4 °C for 30 minutes.

After the supernatant was discarded, the sediment was washed with 1 ml of 70% ethanol, followed by centrifuged at 13,500 rpm at 4 °C for 5 minutes. The top of the aqueous solution was carefully removed, and the pellet was dried in a vacuum for 15 minutes. Afterward, the dried pellet was resuspended in 30 µl of sterile distilled water (DW) and kept at -20 °C. The DNA concentration and purification were analyzed using a microvolume spectrophotometer (DenoVix ® DS-11, DeNovix Inc., Wilmington, DE, USA).

**3.1.1.2 Positive control for SNPs genotyping assay**

The DNA samples for which the genotype was known for the SNP were amplified using the specific primer and conventional Polymerase Chain Reaction (PCR) using the specific primers of each SNP. The primer sequences of each SNP are described below (Table 1) The following components were added for each reaction (Table 2). The thermal cycling conditions and primers are described in Table 3.

**Table 1.** Forward and reverse primer sequences of rs852900542 and rs851998024

| **rs852900542** | |
|---|---|
| Forward primer | 5′-CTCTTCCTCCTGCTCGGATC-3′ |
| Reverse primer | 5′-CGGGTAGGGACCACTGGCAA-3′ |
| **rs851998024** | |
| Forward primer | 5′-GTCAGTGATGTGGCCAAAGGTA-3′ |
| Reverse primer | 5′- TGTGCCTCATCAGGGTCTATG -3′ |

**Table 2.** PCR mixture (KOD Plus Neo, Toyobo Inc., Osaka, Japan) for SNPs genotyping assay

| **Component** | **Volume (µl)** |
|---|---|
| 10x buffer | 2.5 |
| 2mM dNTP | 2.5 |
| 25mM $MgSO_4$ | 1.5 |
| 10µM Forward primer<br>10µM Reverse primer | 0.75 |
| KOD-Plus NEO | 0.5 |
| DNA template | 1 |
| DW | 16.25 |
| **Total volume** | **25** |

**Table 3**. The thermal cycling conditions and primers for PCR

| Step | Temperature (°C) | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | |
| Denaturation | 95 | 30 sec | 40 |
| Annealing | 60* or 62# | 30 sec | |
| Extension | 72 | 30 sec | |
| Final extension | 72 | 5 min | |

* annealing temperature for rs852900542; # annealing temperature for rs851998024

The PCR amplicons were analyzed using 2% agarose gel electrophoresis for 40 minutes. The selected bands were cut from agarose gel and extracted by using a commercial gel extraction kit (RBC HiYield™ Gel/PCR DNA Fragment Extraction Kit, RBC Biosciences, Taipei, Taiwan). The agarose gel containing DNA fragments was excised into small pieces and transferred into a microcentrifuge tube. The 500 µl of DF buffer was added and incubated at 55 °C for 10-15 minutes until the gel piece was completely dissolved. Next, the DF column was placed into a collecting tube. The sample mixture was applied into column and centrifuged at 13,000 rpm for 30 seconds. The flow-through was immediately discarded and 600 µl of wash buffer (ethanol added) to DF column. The mixture was centrifuged at 13,000 rpm for 30 seconds, then discarded the flow-through. For Tris-acetate-EDTA (TAE) gel, the column was repeatedly centrifuged for 2 minutes at 13,000 rpm to dry the column

matrix. The dry column was transferred into a new centrifuge tube. The elution buffer was added about 20-50 µl to the center of the column matrix. Allowed the reaction to stand for 2 minutes until the elution buffer was absorbed by the matrix. The mixture tube was centrifuged for 2 minutes at 13,000 rpm to elute purified DNA. Finally, the purified DNA was kept at -20 °C until further analysis.

**3.1.1.2.1 DNA ligation**

The PCR products were inserted into a plasma vector. The reaction was performed in a microcentrifuge tube (Table 4) and incubated at 4 °C overnight.

**Table 4.** Ligation mixture (pGEM-T easy vector system, Promega Inc., Madison, WI, USA) setup for DNA ligation

| Component | Volume (µl) |
|---|---|
| 2X Ligation buffer | 2 |
| pGEM®-T Easy Vector | 1 |
| T4 DNA ligase (5 units/µl) | 2 |
| DW | 1.5 |
| Purified PCR product | 3.5 |
| **Total volume** | **10** |

**3.1.1.2.2 Transformation**

The plasmid DNA was transformed into *Escherichia coli (E. coli)*–containing ampicillin-resistant gene using the heat shock method (Froger and Hall, 2007). The 50 µl of competent cells (*E. coli*) and 10 µl ligation mixture were mixed on ice in a microcentrifuge tube for 30 minutes, then incubated at 42 ºC for 90 seconds and put back on ice for 2 minutes. The 950 µl of Super Optimal broth with Catabolites repression (SOC) medium, 10 µl of 2M $Mg^{2+}$ ,and 10 µl of 2M glucose were added and shaken in an incubator at 300 rpm, 37 ºC for 90 minutes, followed by centrifuging at 4,000 rpm for 3 minutes. The supernatant was discarded. The 50 µl of cells were spread onto Luria-Bertani (LB) agar plate containing ampicillin and incubated at 37 ºC for 16 to 18 hours. The white colony was selected from LB agar plate using a sterilized loop and inoculated in 2 ml LB broth containing ampicillin. The tube was cultured in an incubated orbital shaker at 37 ºC for 18 hours.



**3.1.1.2.3 Plasmid Extraction**

The colonies of *E. coli* were harvested from LB broth by centrifugation at 13,000 rpm for 1 minute. The plasmid DNA in *E. coli* was isolated by a commercial plasmid DNA purification kit (RBC Real Genomics HiYield™ Plasmid Mini Kit, RBC Biosciences, Taipei, Taiwan). The bacterial culture was transferred into a 1.5 ml microcentrifuge tube and centrifuged at 13,000 rpm for 1 minute. The supernatant was discarded. The 200 µl of $PD_1$ buffer (RNase A added) was added and

resuspended by vortexing or pipetting. The $PD_2$ volume of 200 µl was added and gently mixed by inverting the tube 10 times and standing for 2 minutes at room temperature. Then, 300 µl of PD3 buffer was added and gently mixed by inverting the tube 10 times, followed by centrifugation at 13,000 rpm for 2 minutes. Meanwhile, the PD column was placed into collecting tube. The supernatant was applied into PD column and centrifuged at 13,000 rpm for 30 seconds. The flow-through was discarded. The PD column was returned to collecting tube and added 400 µl of $W_1$ buffer, then centrifuged at 13,000 rpm for 30 seconds. The flow-through was discarded and PD column was replaced to collecting tube. The 600 µl of wash buffer (ethanol added) was added into PD column and centrifuged at 13,000 rpm for 30 seconds. The flow-through was discarded. The PD column was returned to collecting tube. To dry the column, the tube was centrifuged again for 3 minutes at 13,000 rpm. The 50 µl of elution buffer was added directly onto the center of the membrane and stood for 2 minutes until the liquid was absorbed. Finally, the plasmid DNA was eluted by centrifugation at 13,000 for 2 minutes and kept at -20 °C.



**3.1.1.2.4 Colony PCR**

To determine the presence or absence of insert DNA in the plasmid, the primers were designed for specifically target DNA. All PCR reaction components and conditions are described below (Table 5, 6).

**Table 5.** PCR mixture for colony PCR assay

| Components | Volume (µl) |
|---|---|
| 10x buffer | 2.5 |
| 10mM dNTPs mixture | 2.5 |
| 10µM Forward primer<br>10µM Reverse primer | 0.75 |
| Taq DNA polymerase (5 units/µl) | 0.5 |
| $MgSO_4$ | 1 |
| DW | 16.75 |
| LB broth | 1 |
| **Total volume** | **25** |

**Table 6.** The thermal cycling conditions and primers for colony PCR

| Step | Temperature (°C) | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | |
| Denaturation | 95 | 30 sec | 40 |
| Annealing | 60 | 30 sec | |
| Extension | 72 | 30 sec | |
| Final extension | 72 | 3 min | |



The PCR amplicons were resolved using 2% agarose gel electrophoresis for 40 minutes. The selected bands were cut from agarose gel and extracts by using a PCR

gel extraction kit (RBC HiYield™ Gel/PCR DNA Fragment Extraction Kit, RBC Biosciences, Taipei, Taiwan).

**3.1.1.2.5 Nucleotide sequencing analysis**

The nucleotide sequence of positive control was verified at 1st BASE DNA sequencing services, Singapore. The nucleotides of the DNA sequence were compared in the Basic Local Alignment Search Tool (BLAST).

**3.1.1.3 TaqMan Allelic discrimination assay for SNPs detection**

Genotypic analysis of *VDR* gene polymorphisms (rs852900542 and rs851998024) was performed using TaqMan real-time PCR assay (Thermo Fisher Scientific, Waltham, MA, USA) that one (allele T) labeled with FAM dye and the other (allele C) with VIC dye. The intensities of each probe will be plotted in an allelic discrimination graph (Figure 3).

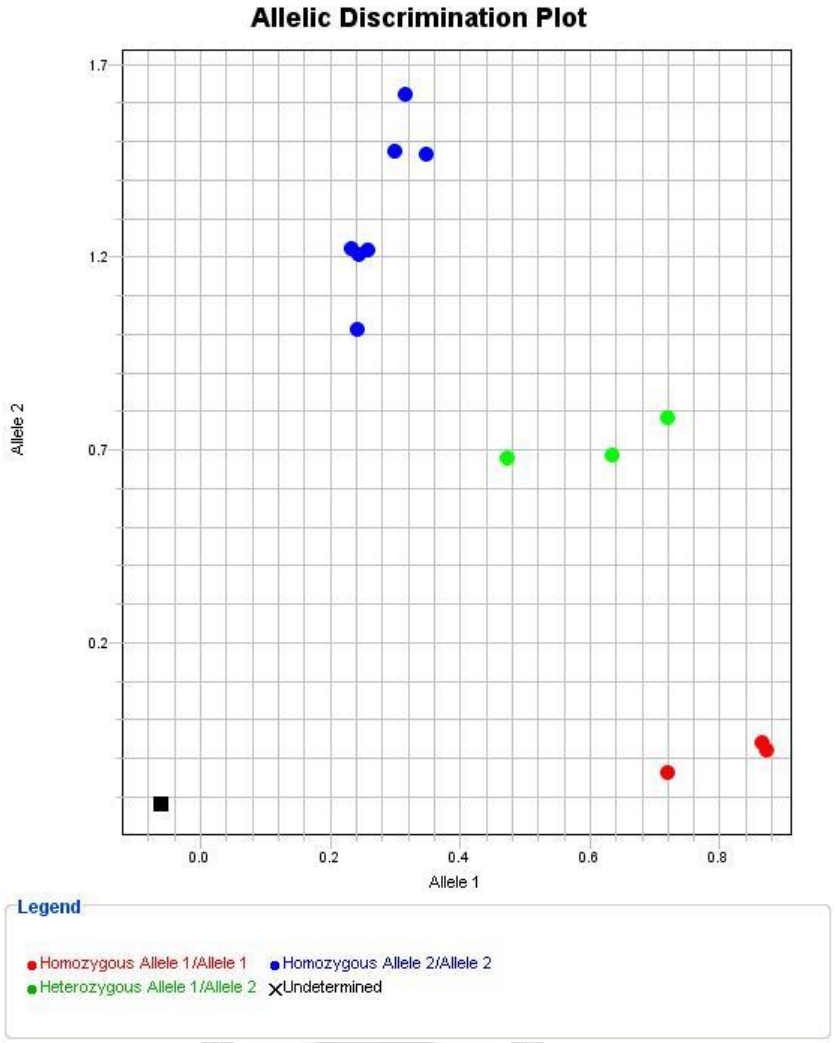




**Figure 3.** The allelic discrimination plot for the rs852900542 SNPs on real-time PCR system. Allele C is plotted for each sample as red circle on the X-axis, and Allele T is plotted as blue circle on the Y-axis. While the heterozygotes are clustered in green in the middle of the plot.

### 3.1.2 Analysis of plasma and urine electrolytes

The concentrations of Ca and P in plasma and urine were measured using automated analyzer (The IL ILab 650 Chemistry Analyzer, Diamond Diagnostic, MA, USA) while Mg was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) Optima 5400 (Perkin Elmer Optima, Waltham, MA, USA). Plasma and urinary creatinine levels were measured using an automated analyzer (The IL ILab 650 Chemistry Analyzer, Diamond Diagnostic, MA, USA). The urinary excretion rate of each mineral is presented as per creatinine ratio.

### 3.1.3 Determination of serum 1,25-dihydroxyvitamin D (1,25(OH)2D)

Quantitative of serum 1,25(OH)2D was performed by canine competitive direct enzyme-linked immunosorbent assay (ELISA) (MBS734600, MyBioSource, San Diego, CA, USA). The 100 µl of standard solutions or samples were added into the well coated with polyclonal anti-1,25(OH)2D antibody, while 100 µl of phosphate buffer solution (PBS) pH 7.0-7.2 was added as blank control. The 50 µl of 1,25(OH)2D-horseradish peroxidase-conjugated was added to each well and mixed well. Afterward, the plate was covered and incubated at 37 $^{o}$C for 1 hour. The incubation mixture was removed by aspiration and subsequently washed the wells five times with washing solution. To dry the blot, the plate was inverted and hit onto absorbent paper. The 50 µl of substrate A and 50 µl of substrate B containing 3, 3', 5, 5'-tetramethylbenzidine (TMB) were added to each well including blank control well.

The plate was covered to protect from sunlight. Later, the samples were incubated at room temperature for 15-20 minutes. Finally, 50 µl of stop solution containing sulfuric acid was added to each well. The absorbance of the samples was determined at 450 nm using a microplate reader immediately. The concentration of serum 1,25-(OH)2D in each sample was calculated by interpolation from the standard curve. The intensity of the color is inversely proportional to the 1,25(OH)2D concentration.

**3.1.4 Statistical analysis**

Data were presented as mean ± standard error (SE). Chi-square or Fisher's exact test was used to determine genotype and allele distribution between the case and control groups. The breed was used as a covariate in the regression for breeds with >3 dogs per group. The breeds with 3 or fewer dogs per group were combined as "other" in the breed category. Possible determinants for calculi risk factors were assessed through generating multiple logistic regression using the dichotomy data of stone status as outcomes and genotype, breed, sex, and age as variables.



The results were expressed in the form of adjusted odds ratios (ORs) with 95 % confidence intervals (CIs). When performing linear regression analysis, the UCa/Cr value was log-transformed and was dependent variables. The independent variables were stone status and genotype. The relation between variables was assessed using R values. Student's t-test or Mann-Whitney U test was used to compare the mean

of variables between groups. All analyses were performed using the commercial SPSS Statistics 22 software (IBM Corp., Armonk, NY, USA). $P<0.05$ was set as statistically significance.

**3.2 Study part II:**

**3.2.1 Sample preparation for proteomic analysis**

Approximate 15 ml of urine samples were collected by free voiding from all dogs. To remove cellular debris, the urine samples were centrifuged at 1000 × g for 10 min. The supernatant was collected and fractionated as aliquots in 1.5 ml tubes and kept at -80 °C until analysis.

Proteomic analysis was performed from urine samples of hypercalciuric dogs in both case and control groups. Hypercalciuria was defined when UCa/Cr≥0.05 as described previously (Furrow et al., 2015).

**3.2.2 Bradford assay for urine protein determination**



Urine protein concentration was determined by Bradford assay (Bradford, 1976). The standard protein was prepared using bovine serum albumin (BSA) in DW and DW was used as blank. While 20 µl of urine sample was diluted to 200 µl with DW. The 1 ml of Bradford reagent was added into each tube and incubated in a dark room for 10 minutes at room temperature. The 200 µl of each sample was transferred in triplicate into a 96-well microtiter plate. The absorbance at 595 nm was measured using a microplate reader (Synergy™ HTX Multi-Mode Microplate

Reader, BioTek Instruments Inc., Vermont, USA) and the protein concentration was determined by comparison with the standard curve.

**3.2.3 Urine protein excretion estimation**

To estimate the rate of protein excretion, the urine protein to creatinine (UPC) ratio was calculated in each dog by dividing the urine protein concentration (mg/dl) by urine creatinine (mg/dl) concentration.

**3.2.4 Urine protein precipitation**

The volume of urine sample from each dog was calculated using equal UPC of several different urine specimens. For protein precipitation, 100% ethanol was added to urine sample (5:1) and incubated at 4 ºC for 10 minutes followed by centrifugation at 12,000 x g for 10 minutes. The supernatant was removed, and the pellet was dissolved in lysis buffer (8M urea in 100 mM triethylammonium bicarbonate; TEAB and sodium dodecyl sulfate; SDS) then kept at 4 ºC.



**3.2.5 Protein separation by one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

Urine protein was separated by SDS-PAGE. The reagents for setting the resolving and stacking gel are shown below (Table7, 8).

**Table 7.** Solutions for preparing 10% resolving gel for SDS-PAGE

| Component | Volume (ml) |
|---|---|
| Deionized Water | 4.830 |
| 40% Acrylamide/Bis | 2.500 |
| 1.5 M Tris-HCl pH 8.8 | 2.500 |
| 10% SDS | 0.100 |
| 10% Ammonium Persulfate (APS) | 0.050 |
| TEMED | 0.005 |

**Table 8.** Solutions for preparing 3.75% Stacking Gel for SDS-PAGE

| Component | Volume (ml) |
|---|---|
| Deionized Water | 1.000 |
| 40% Acrylamide/Bis | 0.154 |
| 0.5 M Tris-HCl pH 6.8 | 0.400 |
| 10% SDS | 0.016 |
| 10% Ammonium Persulfate (APS) | 0.020 |
| TEMED | 0.002 |



The protein sample was mixed with loading dye and heated at 95°C for 5 minutes, and then loaded. The molecular weight standard (ExactPro Broad Range Prestained Protein Ladder, 1st BASE, Singapore) was loaded in control well.

Electrophoresis was performed at 60 volt for 15 minutes in stacking gel and 130 volts for 80 minutes in separating gel. The protein bands were stained with Coomassie Brilliant Blue G250 for 15 minutes. Then, the gel was de-stained overnight and washed with DW. The gel image was taken by gel imaging system (ChemiDoc™ Imaging Systems, Bio-Rad, California, USA).

**3.2.6 Liquid chromatography-mass spectrometry/ mass spectrometry (LC-MS/MS) for protein identification**

**3.2.6.1 In-gel digestion**

**3.2.6.1.1 Coomassie stain and SDS removal**

The bands of interest were excised into small gel pieces (1 - 2 mm) with a clean scalpel, and then placed into 1.5 ml autoclaved pre-lubricated centrifuge tubes. The 200 µl of 25 mM $NH_4HCO_3$ was added for removal of SDS and Coomassie stain and vortexed for 10 minutes. The supernatant was discarded using a gel loading pipet tip. The $NH_4HCO_3$ was added and discarded until the gel pieces were colorless, then left gel pieces overnight at 4 °C. The remaining solvent was removed, and 100% acetonitrile (ACN) was added to cover the gel pieces. The gel pieces were shrunk and became white, then dried in a speed vacuum for 10 minutes.



**3.2.6.1.2 Reduction of disulfide bonds and alkylation of cysteines**

The gel pieces were re-hydrated with 40 µl of 10 mM dithiothreitol (DTT) in 25 mM $NH_4HCO_3$ and allowed the reaction to proceed at 56 °C for 45 minutes. The supernatant was removed, and 10 mM iodoacetamide (IAA) was immediately added to the gel pieces and left for 30 minutes at room temperature in the dark room. After discarded the supernatant, 100 µl of 25 mM $NH_4HCO_3$ in 50% ACN was added to wash the gels for 10 minutes. The solvent was removed followed by adding 200 µl of 100% ACN to shrink the gel pieces. The excess ACN was removed, and then the gel pieces were dried in a speed-vacuum for 10 minutes.

**3.2.6.1.3 Enzymatic digestion**

The fresh trypsin solution was added enough to cover the gel pieces (about 5 - 25 µl) on and incubated on ice or 4 °C for 60 minutes. An excess of trypsin was discarded and 25 mM $NH_4HCO_3$ was added to completely cover the gel pieces, then spun briefly and incubated at 37 °C overnight.



**3.2.6.1.4 Extraction of peptides**

The solution was spun down and kept the supernatant (water extract) into 1.5 ml Protein LoBind tube (Eppendorf, Germany). The 20–30 µl of 0.1% formic acid (FA) in water was added to gel pieces then vortexed and spun. The supernatant (organic extract) was aspirated and combined with the water extract. The gel slice was covered with 0.1% FA in water and placed in a floating rack in a sonicating water bath for 5 minutes before spinning down. The solvent after spinning was kept as the

peptide extracts to a combining tube. The volume of peptide extracts was reduced using speed-vacuum and stored at -20 °C until further analysis.

**3.2.6.2 Peptide desalting method for proteome analysis**

The peptide sample was dissolved in 100 µl of 0.1% FA. Meanwhile, 2 mg of Jupiter C18 (10 mg/1mL of ACN) was loaded onto C18 filter StageTip column and centrifuged at 1,500 x g for 3 minutes. To equilibrate column tip, 100 µl of 0.1% FA was filled and centrifuged at 1,500 x g for 3 minutes. The peptide sample was loaded into column and centrifuged at 3,000 x g for 3 minutes. This solution was kept as a flow-through fraction. Then, the column was washed by adding 100 µl of 0.1% FA and centrifuged at 3,000 x g for 3 minutes to keep this fraction as a washed fraction. The 100 µl of 0.1% FA/50% ACN was added to elute peptides in column, then centrifuged at 1,500 x g for 3 minutes. This fraction was kept in Protein LoBind tube as eluted peptide fraction and dried in a speed-vacuum before kept at -80 °C until Mass spectrometric analysis.



**3.2.6.3 LC-MS/MS analysis**

The peptides from eluted peptide fraction were analyzed by a nano-flow liquid chromatography (EASY-nLC 1000 Liquid Chromatograph, Thermo Fisher Scientific, MA, USA) coupled to a mass spectrometer (Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer, Thermo Fisher Scientific, MA, USA). through an EASY-Spray™ Sources (Thermo Fisher Scientific, MA, USA).

Raw data files from the mass spectrometer were used to search against a canine protein database using SEQUEST HT™ database searching algorithms, which is part of the Proteome Discoverer software (Thermo Scientific™ Proteome Discoverer™ software v.2.1.1.21, Thermo Fisher Scientific, MA, USA). The protein sequences and functional information of specific proteins were used in the annotation of UniProtKB/Swiss-Prot entries (https://www.uniprot.org/).

### 3.2.7 Statistical analysis

Transforming data of unique proteins in logarithm was performed. The relative ratio or fold-change of each protein was compared between hypercalciuric stone-free and stone former dogs. Unpaired t-test was used to compare the differences in protein abundances between groups. $P<0.05$ was set as statistically significance.

# CHAPTER IV

# RESULTS

## 1. Study part I

### 1.1 Characteristics of the study population

Seventy-five dogs were enrolled in this study. The characteristics of the study population are shown in Table 9. There were six breeds of dogs, including Pomeranian, Shih-Tzu, Chihuahua, Schnauzer, Yorkshire Terrier, and Maltese. Forty control dogs consisted of 22 males (intact male; M=11, castrated male; Mc=11) and 18 females (intact female; F=10, spayed female; Fs=8). While thirty-five cases were composed of 26 males (M=9, Mc=17) and 9 females (F=4, Fs=5). The sex distributions between the cases and controls were not significantly different (P=0.14). The average age of the dogs in the case group was significantly higher than that of the controls (9.0±3.0 VS 7.3±3.0, P<0.05). The compositions of the stone body in all cases were constituted by 100% CaOx (dihydrate + monohydrate). Nine Pomeranians, nine Shih Tzus, and five Chihuahuas had a stone body composition of CaOx monohydrate (≥70%). The stone type of the other breeds (n=7) was composed of 100% CaOx monohydrate. Eleven of the 35 cases (31.4%) were recurrent CaOx stone formers. Most CaOx stones occurred with greater frequency in the lower urinary tract (91.4 %).

**Table 9.** Characteristics data of study population

| Characteristics | Control (n=40) | Case (n=35) |
|---|---|---|
| **Sex** | | |
| Males [M/Mc] | 22 [11/11] | 26 [9/17] |
| Females [F/Fs] | 18 [10/8] | 9 [4/5] |
| **Age** (years) | 7.3±0.5 | 9.0±0.5* |
| **Breed** | | |
| Pomeranian | 19 (47.5%) | 12 (34.3%) |
| Shih Tzu | 11 (27.5%) | 10 (28.6%) |
| Chihuahua | 6 (15%) | 6 (17.1%) |
| Schnauzer | 1 (2.5%) | 3 (8.6%) |
| Yorkshire Terrier | 2 (5%) | 2 (5.7%) |
| Maltese | 1 (2.5%) | 2 (5.7%) |
| **Episode** | | |
| First | - | 24 (68.6%) |
| Second | - | 11 (31.4%) |
| **Location** | | |
| Upper urinary tracts (kidney, ureter) | - | 3 (8.6%) |
| Lower urinary tracts (urinary bladder, urethra) | - | 32 (91.4%) |

Age presented as mean ± standard error (SE)

* indicates statistically difference at P<0.05

M: intact male; Mc: castrated male; F: intact female; Fs: spayed female

CBC and serum blood chemistry profiles in each group are shown in Table 10. There was no difference in CBC between groups. Serum ALT, BUN, Cr, TP ,and ALB were also not different between groups. However, ALP level was significantly higher in case groups compared with controls (92.9±8.2 VS 67.7±8.5; P<0.01). Results of urinalysis were presented in Table 10. The case had significantly lower urine pH compared with controls (5.3±0.1 VS 7.0±0.2; P<0.01). There was no difference in specific gravity between groups.

**Table 10.** Complete blood count (CBC), serum blood chemistry profiles, and urinalysis in each experiment groups

| Variables | Control (n=40) | Case (n=35) |
|---|---|---|
| **CBC** | | |
| Red blood cells (x$10^6$ cell/ul) | 6.9±0.2 | 6.5±0.1 |
| Hemoglobin (g/dl) | 16.1±0.4 | 15.3±0.4 |
| Hematocrit (%) | 45.0±1.0 | 42.4±1.0 |
| Platelets (x$10^3$ cell/ul) | 291.4±12.4 | 335.4±18.8 |
| White blood cells (x$10^3$ cell/ul) | 12.8±2.5 | 9.2±0.4 |
| **Blood chemistry** | | |
| ALT (unit/l) | 52.3±3.5 | 49.3±6.6 |
| ALP (unit/l) | 67.7±8.5 | 92.9±8.2** |
| BUN (mg/dl) | 20.6±1.4 | 17.9±1.6 |
| Cr (mg/dl) | 0.90±0.04 | 0.80±0.04 |
| TP (g/dl) | 7.0±0.2 | 6.8±0.2 |
| Albumin (g/dl) | 3.4±0.1 | 3.3±0.1 |
| **Urinalysis** | | |
| Specific gravity | 1.027±0.003 | 1.032±0.002 |
| pH | 7.0±0.2 | 5.3±0.1** |

Data were presented as mean ± standard error (SE)

** indicates statistically difference at P<0.01

**1.2 Serum electrolyte and vitamin D levels**

Serum electrolyte levels are shown in Table 11. All dogs had serum calcium levels within the reference interval. Serum calcium levels were significantly higher in CaOx dogs than in controls (9.92±0.24 VS 8.18±0.19 mg/dl, P<0.01). The cases had significantly lower serum phosphate compared with controls (P<0.05). No significant differences in serum magnesium levels were found between the cases and the controls. There was no significant difference in serum creatinine concentration (control = 0.90±0.04 mg/dl, case = 0.80±0.04 mg/dl, P=0.07) and 1,25-(OH)2D levels between the cases (308.7±39.7 pg/ml) and controls (286.7±39.5 pg/ml) (P=0.45).

**Table 11.** Comparison of serum electrolytes and 1,25(OH)2D concentration in controls and CaOx cases

| Variables | Control (n=40) | Case (n=35) |
|---|---|---|
| **Serum electrolytes (mg/dl)** | | |
| Calcium | 8.18±0.19 | 9.92±0.24*** |
| Phosphate | 4.31±0.21 | 3.96±0.37* |
| Magnesium | 2.35±0.11 | 2.38±0.17 |
| Creatinine | 0.90±0.04 | 0.80±0.04 |
| **1,25(OH)2D (pg/ml)** | 286.7±39.5 | 308.7±39.7 |

Results presented as mean ± standard error (SE)

* indicates statistically difference at P<0.05

*** indicates statistically difference at P<0.001

**1.3 Urinary electrolyte excretion between cases and controls**

Spot urinary electrolytes were normalized with urinary creatinine in each dog (Table 12, Figure 4). Regarding urinary excretion rate, dogs in the case group had significantly higher UCa/Cr (control = 0.056±0.009 VS case = 0.083±0.010, P<0.05), and UMg/Cr Cr (control = 0.063±0.008 VS case = 0.104±0.012, P<0.05) than those in controls. However, there was no difference in UP/Cr between these groups.

**Table 12.** Spot urinary electrolyte to creatinine ratio in dogs with CaOx history (cases) and stone-free dogs (controls)

| Variables | Control | Case |
|---|---|---|
| **Urinary excretion of electrolytes** | | |
| UCa/Cr | 0.056±0.009 | 0.083±0.010* |
| UP/Cr | 1.15±0.19 | 1.26±0.13 |
| UMg/Cr | 0.063±0.008 | 0.104±0.012* |



Results presented as mean ± standard error (SE)

* indicates statistically difference at P<0.05

UCa/Cr: urinary calcium to creatinine ratio; UP/Cr: urinary phosphate to creatinine ratio; UMg/Cr: urinary magnesium to creatinine ratio

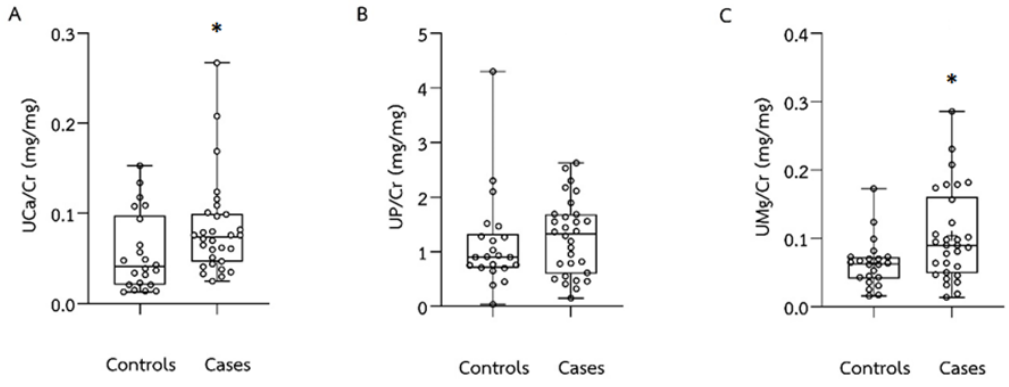


**Figure 4.** Box and whisker plots of spot urinary calcium-to-creatinine ratio (A) (UCa/Cr), urinary phosphate-to-creatinine ratio (B) (UP/Cr), and urinary magnesium-to-creatinine ratio (C) (UMg/Cr) in dogs with CaOx stone formation (cases) and dogs (controls). Each open circles represent individual dog measurement.

* indicates statistically difference at P<0.05.

**1.4 VDR SNPs Distribution in Controls and Urolithiasis Dogs**

We analyzed the SNP distribution and allele frequency of rs852900542 and rs851998024 in the *VDR* gene in 40 controls and 35 dogs with CaOx urolithiasis. The genotype distribution of each SNP in the study population did not deviate from the Hardy–Weinberg equilibrium (P<0.05) (https://scienceprimer.com/hardy-weinberg-equilibrium-calculator).

### 1.4.1 rs852900542 genotype distribution

Our results showed that three genotypes of rs852900542 were identified including TT (homozygous wild type), CT (heterozygous), and CC (homozygous mutant) (Table 13, Figure 5). Dogs in case groups were 19 homozygous for TT genotype (54%), 10 (28.6%) heterozygous and 6 (17.1%) homozygous for CC genotype. While dogs in control group were 31 (77.5%), 8 (20%) and 1 (2.5%) with TT, CT, and CC genotype, respectively. A significant difference was found in the distribution of genotypes between CaOx stone and control dogs ($P<0.05$).

The major allele that was found in this study population was the T allele. There was a significant difference in allele frequency between the CaOx dogs and controls ($P< 0.05$). In control group, the major allele (T) frequency was 87.5% and minor allele (C) frequency was 12.5%. Whereas the allele frequencies were 68.6% for the T allele and 31.4% for the C allele in CaOx dogs. The allele distribution of rs852900542 was presented below (Table 14, Figure 6).

**Table 13.** Genotype frequencies of rs852900542 in dogs with history of CaOx (cases) and breed-matched stone-free dogs (controls).

| Genotype distribution (%) | Control (n=40) | Case (n=35) | *p*-value |
|---|---|---|---|
| **rs852900542** | | | 0.04* |
| TT | 31 (77.5) | 19 (54.3) | |
| CT | 8 (20.0) | 10 (28.6) | |
| CC | 1 (2.5) | 6 (17.1) | |
| CT+CC | 9 (22.5) | 16 (45.7) | |

* indicates statistically difference at P<0.05 using Fisher's Exact test compared between TT and CT+CC genotype

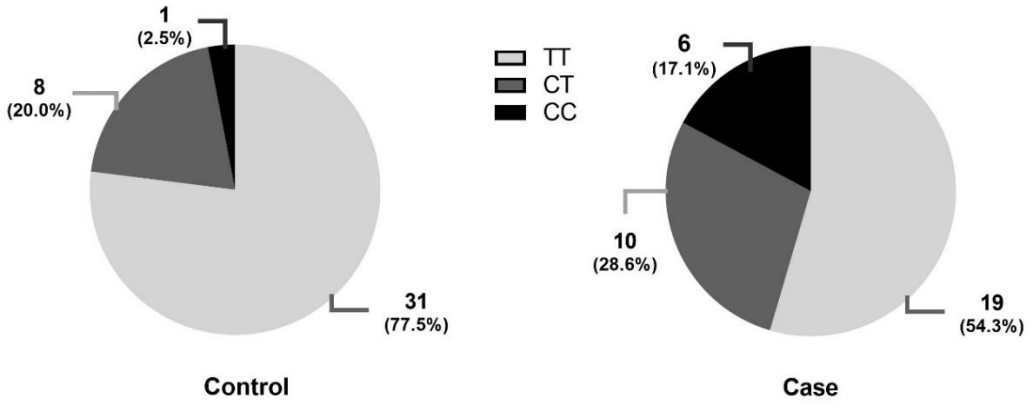


**Figure 5.** Genotypic frequencies of rs852900542 *VDR* gene in CaOx case and control dogs in the study

**Table 14.** Allele frequencies of rs852900542 in dogs with history of CaOx (cases) and breed-matched stone-free dogs (controls)

| Allele frequencies (%) | Control (n=40) | Case (n=35) | P-value |
|---|---|---|---|
| T | 70 (87.5) | 48 (68.6) | 0.01* |
| C | 10 (12.5) | 22 (31.4) | |

* indicates statistically difference at P<0.05

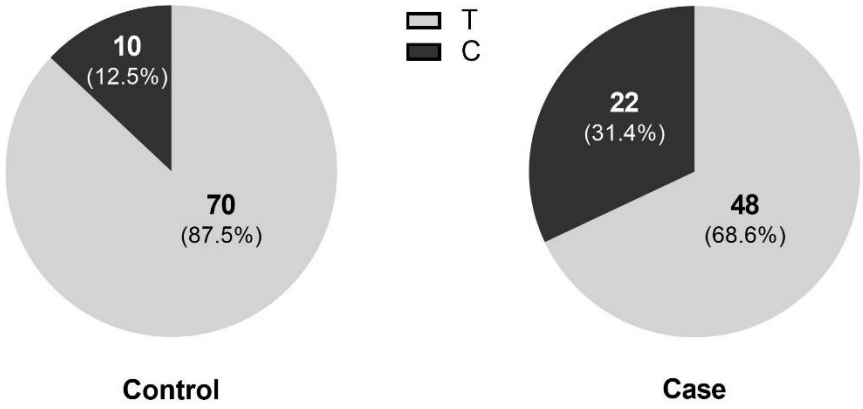


**Figure 6.** Allele frequencies of rs852900542 *VDR* gene in CaOx case and control dogs in the study

### 1.4.2 rs851998024 genotype distribution

For rs851998024 distribution, there was only the homozygous TT genotype identified in dogs in this study and was not associated with CaOx stone risk in this population samples.

### 1.5 Influence of rs852900542 genotype on urolithiasis risk in dogs

We defined the influence of rs852900542 genotypes on urolithiasis risk using multiple logistic regression (Table 15), the result showed that dogs with a CC or CT genotype had an increased risk for CaOx stones than those with the TT genotype (OR = 3.82, 95% CI 1.04 – 13.98, P<0.05). Moreover, the risk of CaOx urolithiasis correlated with increasing age (OR = 1.25, 95% CI 1.04 - 1.50, P<0.05).

**Table 15.** Multiple logistic regression analysis to determine factors associated with CaOx urolithiasis



| | Adjusted OR | 95% CI | P-value |
|---|---|---|---|
| **rs852900542 genotype** | | | |
| CT and CC VS TT | 3.82 | 1.04-13.98 | 0.04* |
| **Breed**[#] | 0.78 | 0.38-1.64 | 0.52 |
| **Sex** | 0.48 | 0.15-1.50 | 0.20 |
| **Age** | 1.25 | 1.04-1.50 | 0.02* |

* indicates statistically difference at P<0.05; OR: Odds ratio; CI: confident interval

# including three main breeds in study population (Pomeranian, Shih Tzu, and Chihuahua)

The case dogs were subsequently categorized into two subgroups, homozygous reference (TT) versus homozygous or heterozygous for variant allele (CC and CT, respectively) (Table 16). There were no significant differences in serum calcium, phosphate, magnesium, creatinine ,and 1,25-(OH)2D concentrations between the subgroups of CaOx dogs.

**Table 16.** Comparison of serum electrolytes and 1,25(OH)2D concentration in CaOx stone-forming dogs according to rs852900542 genotype

| Variables | TT (n=19) | CT and CC (n=11) |
|---|---|---|
| **Serum electrolytes (mg/dl)** | | |
| Calcium | 10.17±0.31 | 9.48±0.35 |
| Phosphate | 3.68±0.20 | 4.45±0.95 |
| Magnesium | 2.49±0.26 | 2.19±0.11 |
| Creatinine | 0.83±0.05 | 0.73±0.05 |
| **1,25(OH)2D (pg/ml)** | 316.9±60.4 | 294.7±33.2 |

Results presented as mean ± standard error (SE)

When considering urinary excretion of electrolytes, CaOx dogs with the CT or CC genotypes had significantly higher UCa/Cr (TT = 0.072±0.009 VS CT+CC = 0.110±0.022, P<0.05), and UMg/Cr levels than those with TT genotype (TT = 0.084±0.012 VS CT+CC = 0.138±0.023, P<0.05) (Table 17, Figure 7). While there was no difference in UP/Cr between subgroups of CaOx dogs (TT = 1.23±0.16 mg/mg VS CT+CC = 1.32±0.21). When considering control group, however, there was no difference in UCa/Cr between TT genotype and CT+CC genotype in control dogs (0.051±0.01 VS 0.067±0.019), as well as in UMg/Cr (0.063±0.009 VS 0.063±0.015) and UP/Cr (1.04±0.24 VS 1.45±0.27) (Figure 8).

**Table 17.** Spot urinary electrolyte to creatinine ratio in dogs with CaOx history (cases) according to rs852900542 genotypes.

| Variables | TT (n=19) | CT and CC (n=11) |
|---|---|---|
| **Urinary excretion of electrolytes** | | |
| UCa/Cr | 0.072±0.009 | 0.110±0.022* |
| UP/Cr | 1.23±0.16 | 1.32±0.21 |
| UMg/Cr | 0.084±0.012 | 0.138±0.023* |

Results presented as mean ± standard error (SE)

* indicates statistically difference at P<0.05

UCa/Cr: urinary calcium to creatinine ratio; UP/Cr: urinary phosphate to creatinine ratio; UMg/Cr: urinary magnesium to creatinine ratio

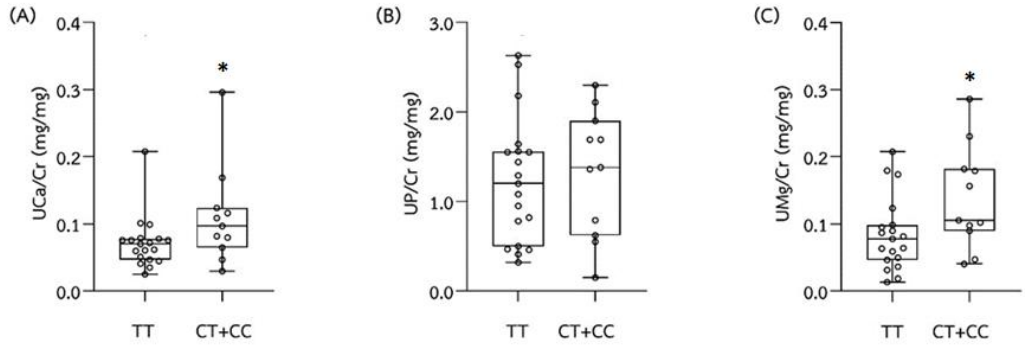


**Figure 7.** Box and whisker plots of spot urinary calcium-to-creatinine ratio (A) (UCa/Cr), urinary phosphate-to-creatinine ratio (B) (UP/Cr), and urinary magnesium-to-creatinine ratio (C) (UMg/Cr) in dogs with CaOx stone formation (cases) carrying TT genotype and non-TT (CT+CC) genotype of rs852900542. Each open circles represent individual dog measurement.

* indicates statistically difference at $P<0.05$.

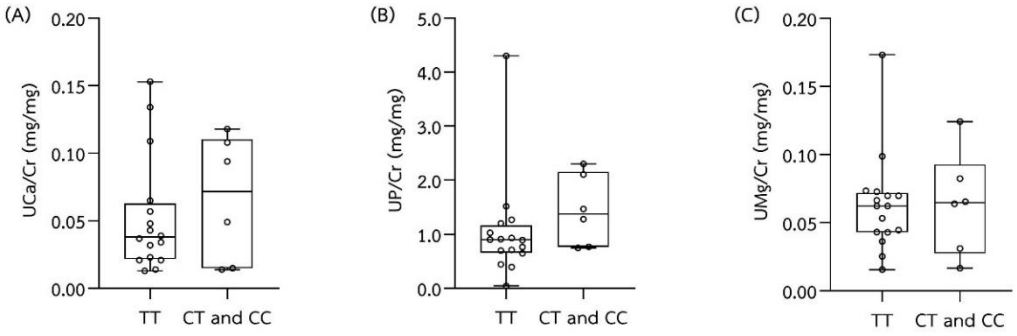


**Figure 8.** Box and whisker plots of spot urinary calcium-to-creatinine ratio (A) (UCa/Cr), urinary phosphate-to-creatinine ratio (B) (UP/Cr), and urinary magnesium-to-creatinine ratio (C) (UMg/Cr) in control dogs carrying TT genotype and non-TT (CT+CC) genotype of rs852900542. Each open circles represent individual dog measurement.

To estimate the influence of stone status and rs852900542 genotype on urinary calcium excretion, UCa/Cr was transformed to log UCa/Cr as the dependent variable, and the genotype plus case-control status as independent variables (Table 18), the results showed that CaOx stone-forming dogs are associated with higher urinary calcium excretion compared with controls (R=0.42, P<0.05). However, genotype had no association with urinary excretion of calcium when considering in all dogs (P=0.12) although the differences of urinary excretion of calcium between CT+CC and TT genotypes were found in case dogs.

**Table 18.** The effect of stone status and rs852900542 genotype on urinary calcium excretion using multiple linear regression analysis

| | B | SE | t | P-value |
|---|---|---|---|---|
| **constant** | -0.99 | 0.13 | -7.78 | <0.001[a] |
| **Group** | -0.21 | 0.08 | -2.64 | 0.01[a] |
| **Genotype (CT and CC)** | 0.13 | 0.08 | 1.57 | 0.12 |

[a] The regression was performed using log UCa/Cr as dependent variable.

B: coefficient; SE: standard error

Log UCa/Cr = - 0.986 - (0.212 × group) + (0.133 × genotype)

R=0.42; F-statistic: 5.16; P=0.01

**2. Study Part II**

**2.1 Characteristics of study population**

Only the dogs from the study I which had urinary calcium to creatinine ratio ≥0.05 were enrolled in study II. The dogs were defined into case (n=7) and control with breed and sex-matched (n=7). The characteristics of the study population are shown in Table 19. There were three breeds of dogs in the study, including Pomeranian, Chihuahua, and Miniature Schnauzer. Control dogs consisted of 6 males (M=3, Mc=3 and 1 female (F=0, Fs=1). While seven cases were composed of 6 males (M=4, Mc=2) and 1 female (F=0, Fs=1). The average age of the dogs in case group was not different from that of the controls.

All serum parameters including calcium, magnesium ,and creatinine were not different between cases and controls (Table 19). Regarding urinary excretion rate, there was no difference between case and control group in UCa/Cr, UMg/Cr, and UPC ratio. However, hypercalciuric CaOx stone-formers had lower urine pH compared with stone-free controls. Urine Specific gravity was not differed between groups.

**Table 19.** Characteristics data, serum, and urinary variables of study population

| Variables | Control (n=7) | Case (n=7) |
|---|---|---|
| **Age** | 7.3±0.8 | 6.4±0.9 |
| **Sex** | | |
| M/Mc | 3/3 | 4/2 |
| F/Fs | 0/1 | 0/1 |
| **Serum electrolytes (mg/dl)** | | |
| Calcium | 8.60±0.46 | 7.86±0.66 |
| Magnesium | 2.32±0.18 | 1.99±0.17 |
| Creatinine | 0.80±0.08 | 0.74±0.11 |
| **Urinary excretion of electrolyte** | | |
| UCa/Cr | 0.10±0.01 | 0.13±0.03 |
| UMg/Cr | 0.06±0.01 | 0.11±0.03 |
| **Urine pH** | 6.6±0.3 | 5.4±0.3* |
| **UPC ratio** | 0.14±0.02 | 0.15±0.07 |
| **Urine Specific gravity** | 1.030±0.002 | 1.035±0.004 |

Results presented as mean ± standard error (SE)

* indicates statistically difference at P<0.05

Cr: creatinine; UPC: urine protein/urine creatinine ratio

### 2.2 Separated urinary proteins by SDS-PAGE gel electrophoresis

An equal amount of urine protein excretion rate or UPC was used to calculate the volume of urine sample from each individual. The urine protein in each sample was separated by electrophoresis and shown in Figure 9.

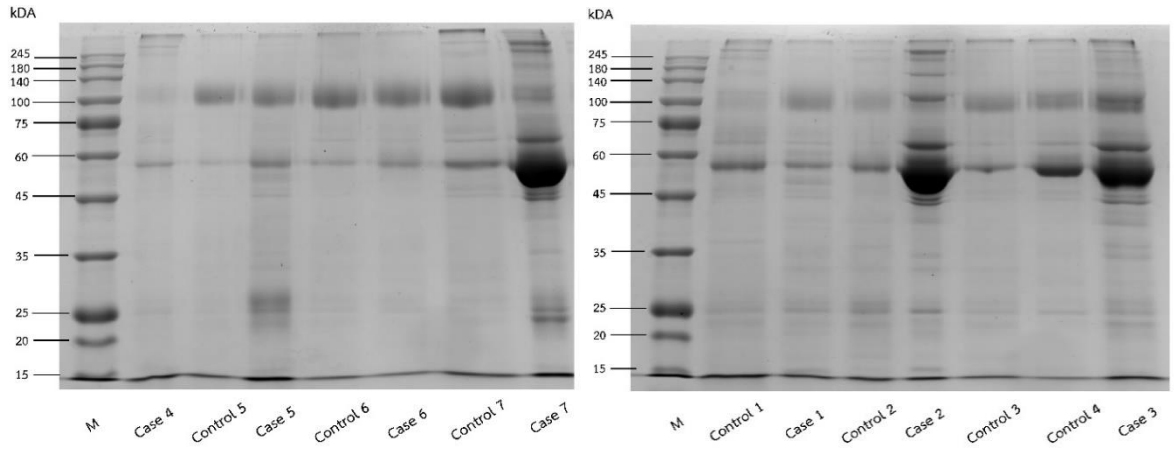


**Figure 9.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of urine proteins in dogs with CaOx stone (case, n=7) and stone-free controls (control, n=7). M = molecular weight marker.



### 2.3 Protein identified in stone-free dogs and CaOx dogs by LC-MS/MS

There were 55 proteins identified in stone-free control dogs while 68 proteins were found in CaOx dogs. Forty-nine proteins were identified in urine from both groups. Nineteen and 6 proteins were exclusively found in CaOx stone former and stone-free urine, respectively (Figure 10). The lists of proteins found in hypercalciuric

stone-free control dogs and hypercalciuric CaOx dogs were shown in Table 20 and 21.

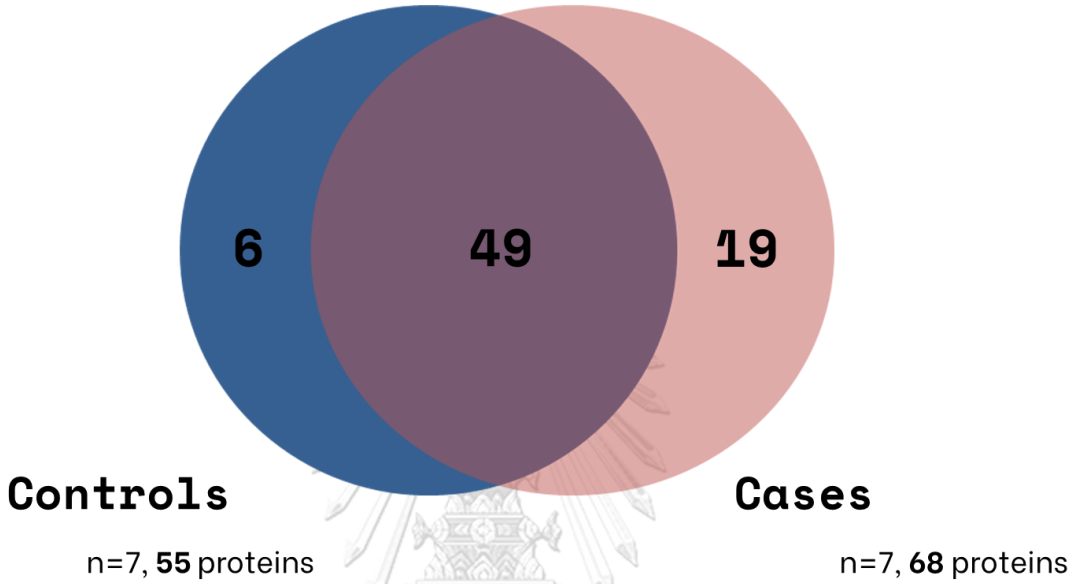


**Figure 10.** Expression of proteins found in stone-free dogs and CaOx stone-former dogs.

**Table 20.** List of urinary proteins identified by LC-MS/MS found only in stone-free dogs

| UniProt ID | Protein Description | Score SEQUEST HT | UqP | Coverage |
|---|---|---|---|---|
| Q71DR4 | Ciliary neurotrophic factor receptor subunit alpha | 13.44 | 2 | 6.451613 |
| Q2PT31 | Myocilin | 6.42 | 3 | 5.175983 |
| Q9GLD3 | Transferrin receptor protein 1 | 3.29 | 2 | 2.597403 |
| P50994 | Annexin A4 | 4.61 | 2 | 9.090909 |
| P48300 | Tissue alpha-L-fucosidase | 5.29 | 2 | 4.731183 |
| P0658 vc3 | Sodium/potassium-transporting ATPase subunit beta-1 | 0 | 2 | 6.930693 |

UqP: Unique Peptides

**Table 21.** List of urinary proteins identified by LC-MS/MS found only in CaOx stone-forming dogs

| UniProt ID | Protein Description | Score SEQUEST HT | UqP | Coverage |
|---|---|---|---|---|
| O18733 | Matrix metalloproteinase-9 | 91.35 | 22 | 35.65341 |
| Q258K2 | Myosin-9 | 4.7 | 8 | 4.693878 |
| E2RE76 | Apolipoprotein A-IV | 14.12 | 4 | 11.11111 |
| Q9GL24 | Procathepsin L | 11.03 | 3 | 11.41141 |
| O18835 | Beta-glucuronidase | 4.12 | 4 | 5.376344 |
| P81709 | Lysozyme C, spleen isozyme | 29.91 | 3 | 27.69231 |
| P68213 | Fibrinogen alpha chain | 15.7 | 3 | 67.85714 |
| Q28894 | WAP four-disulfide core domain protein 2 | 19.44 | 2 | 38.70968 |
| B6V8E6 | Catenin beta-1 | 3.11 | 2 | 3.072983 |
| P18470 | DLA class II histocompatibility antigen, DR-1 beta chain | 5.58 | 2 | 10.15038 |
| P19540 | Coagulation factor IX | 7.07 | 3 | 6.19469 |

| UniProt ID | Protein Description | Score SEQUEST HT | UqP | Coverage |
|---|---|---|---|---|
| Q53VB8 | Ferritin light chain | 9.44 | 2 | 17.71429 |
| Q9GLK0 | Protein-glutamine gamma-glutamyltransferase K | 1.9 | 2 | 1.96319 |
| Q076A6 | Myosin-1 | 0 | 1 | 0.876741 |
| Q9TSZ6 | Dystroglycan | 3.1 | 2 | 3.251121 |
| Q8SQ41 | Pepsin B | 14.07 | 2 | 5.128205 |
| Q076A7 | Myosin-2 | 1.78 | 2 | 1.597938 |
| P51152 | Ras-related protein Rab-12 | 3.96 | 2 | 10.09615 |
| Q5XNR9 | Leukemia inhibitory factor receptor | 2.08 | 2 | 3.281677 |

UqP: Unique Peptides

**Table 22.** The difference of urinary protein levels identified in hypercalciuric stone-free dogs and hypercalciuric CaOx stone-former dogs

| UniProt ID | Protein Description | Fold-change | Molecular function | Biological process | P-value |
|---|---|---|---|---|---|
| Q5W7P8 | Thrombomodulin | 1.8 | endothelial cell receptor | blood coagulation | 0.007 |
| P33729 | Intercellular adhesion molecule 1 | 0.5 | integrin binding | cell adhesion | 0.142 |
| P49256 | Vesicular integral-membrane protein VIP36 | 1.6 | protein binding | protein transport | 0.157 |
| P60524 | Hemoglobin subunit beta | 5.0 | oxygen transport | oxygen transport | 0.165 |
| Q9TSX8 | Pantetheinase | 2.0 | hydrolase | pantothenate metabolic process | 0.167 |
| O46634 | CD166 antigen | 2.1 | Cell adhesion molecule | immunity, cell adhesion | 0.181 |
| Q9BEA0 | Pro-epidermal growth factor | 0.8 | growth factor | receptor signaling pathway | 0.232 |

| UniProt ID | Protein Description | Fold-change | Molecular function | Biological process | P-value |
|---|---|---|---|---|---|
| P01785 | Ig heavy chain V region MOO | 1.4 | antigen binding | immunity | 0.280 |
| O97578 | Dipeptidyl peptidase 1 | 2.2 | protease | proteolysis | 0.286 |
| Q7YQC6 | Heat shock 70 kDa protein 1 | 1.4 | Chaperone | stress response | 0.291 |
| P80009 | Plasminogen | 5.1 | protease | blood coagulation | 0.318 |
| P33703 | Beta-2-glycoprotein 1 | 2.4 | heparin binding | blood coagulation | 0.318 |
| Q9XSB8 | Tripeptidyl-peptidase 1 | 1.0 | protease | proteolysis | 0.325 |
| Q4LAL9 | Cathepsin D | 2.4 | protease | | 0.327 |
| Q28894 | WAP four-disulfide core domain protein 2 | 1.4 | protease inhibitor | immune response | 0.352 |
| Q28256 | Platelet glycoprotein Ib alpha chain | 1.0 | membrane protein | blood coagulation, cell adhesion | 0.366 |
| P01784 | Ig heavy chain V region GOM | 1.3 | antigen binding | immunity | 0.368 |
| Q52S86 | Podocalyxin | 1.7 | anti-adhesive molecule | cell adhesion | 0.380 |

| UniProt ID | Protein Description | Fold-change | Molecular function | Biological process | P-value |
|---|---|---|---|---|---|
| P79143 | Aminopeptidase N | 0.7 | protease | angiogenesis | 0.398 |
| Q9GMY6 | Pepsin A | 0.8 | protease | digestion | 0.415 |
| P06872 | Anionic trypsin | 1.7 | protease | digestion | 0.427 |
| P42929 | Heat shock protein beta 1 | 1.4 | Chaperone | stress response | 0.442 |
| Q6TEQ7 | Annexin A2 | 1.3 | calcium binding protein | | 0.469 |
| P09582 | Arginine esterase | 11.0 | protease | proteolysis | 0.493 |
| Q28259 | Glyceraldehyde-3-phosphate dehydrogenase | 0.9 | transferase | immunity | 0.506 |
| P49822 | Albumin | 1.5 | metal binding | | 0.514 |
| Q8MJU5 | Alpha-fetoprotein | 1.7 | metal binding | | 0.550 |
| P60529 | Hemoglobin subunit alpha | 8.7 | oxygen transport | oxygen transport | 0.556 |
| O18840 | Actin, cytoplasmic 1 | 1.0 | structural constituent | cell motility | 0.630 |

| UniProt ID | Protein Description | Fold-change | Molecular function | Biological process | P-value |
|---|---|---|---|---|---|
| F1PAA9 | Cadherin-1 | 0.8 | adhesion protein | cell adhesion | 0.660 |
| Q862Z3 | Uromodulin | 1.1 | receptor for immune response | neutrophil migration | 0.661 |
| Q6QNF3 | Platelet-derived growth factor receptor beta | 0.8 | platelet binding, growth factor binding | chemotaxis | 0.662 |
| O18873 | Major allergen Can f 1 | 1.8 | small molecule binding | transport | 0.717 |
| Q28275 | Fibronectin | 2.1 | DNA binding, heparin binding | cell adhesion, inflammation | 0.791 |
| Q9TU53 | Cubilin | 0.9 | metal binding | protein transport | 0.802 |
| P02648 | Apolipoprotein A-I | 1.4 | lipid binding | lipid transport | 0.811 |
| Q9GKQ8 | Desmoglein-1 | 1.4 | adhesion protein | cell adhesion | 0.821 |
| P18466 | DLA class I histocompatibility antigen, A9/A9 alpha chain | 0.5 | | immunity | 0.840 |

| UniProt ID | Protein Description | Fold-change | Molecular function | Biological process | P-value |
|---|---|---|---|---|---|
| Q6QNF4 | Hepatocyte growth factor activator | 0.8 | protease | | 0.874 |
| Q28260 | Vascular cell adhesion protein 1 | 0.9 | integrin binding | cell adhesion | 0.884 |
| P25473 | Clusterin | 2.1 | Chaperone | complement cascade | 0.901 |
| Q28284 | CD44 antigen | 0.9 | receptor | cell adhesion | 0.902 |
| Q9XS65 | Prostaglandin-H2 D-isomerase | 1.4 | isomerase | Prostaglandin biosynthesis | 0.908 |
| Q71DR4 | Ciliary neurotrophic factor receptor subunit alpha | 1.3 | receptor | | 0.911 |
| Q8SQ41 | Pepsin B | 0.9 | protease | digestion | 0.916 |
| P19006 | Haptoglobin | 1.3 | antioxidant | acute inflammatory response | 0.950 |

| UniProt ID | Protein Description | Fold-change | Molecular function | Biological process | P-value |
|---|---|---|---|---|---|
| Q6UKI2 | Protein amnionless | 0.8 | | protein transport | 0.973 |
| P48300 | Tissue alpha-L-fucosidase | 1.2 | glycosidase | glycoside catabolic process | 0.988 |
| Q767J3 | Deoxyribonuclease-1 | 1.3 | endonuclease | apoptosis | 0.997 |

# CHAPTER V

# DISCUSSION

## 1. Study part I

In the present study, there were six of small-breed dogs that are predisposed to CaOx urolith, similar to the other studies in which small-breed dogs are at high-risk for CaOx stone formation (Wisener et al., 2010; Hunprasit et al., 2019). This could be due to higher urinary calcium excretion, which contributed to CaOx stone formation in small-breed dogs (Stevenson et al., 2003). However, the mechanism is still unclear. Not surprisingly, CaOx occurs more frequently in middle-aged dogs (Houston et al., 2017; Hunprasit et al., 2017). The higher prevalence of CaOx urolithiasis was observed in male than female dogs, as has been reported previously (Picavet et al., 2007; Okafor et al., 2014; Hunprasit et al., 2017). Male predisposing to CaOx stone formation might be the effect of testosterone on oxalate excretion (Yagisawa et al., 2001; Nath et al., 2013). Additionally, neutered dogs had a high incidence which might be due to weight-gaining susceptibility. While overweight or obesity is strongly associated with risk of CaOx formation and increased urinary excretion in human (Siener et al., 2004; Taylor et al., 2005). However, the effects of these factors on CaOx urolithiasis in dog are not clearly defined.



The serum ALP was significantly higher in CaOx dogs compared with controls, although serum ALP concentration was within the reference interval for dogs. Mild elevation of serum ALP concentration is frequently found after a surgical procedure or is possible from urinary bladder injury (Leibovitch et al., 1991). Dogs in case groups

had high urine acidity which predispose to develop CaOx stone formation similar to previous studies (Lekcharoensuk et al.., 2002; Okafor et al., 2014).

The serum calcium in CaOx dog group was significantly higher than controls but within the normal limits, which was consistent with previous studies (Lulich et al., 1991; Furrow et al., 2015). Moreover, the UCa/Cr ratio was higher in CaOx dogs than that in controls. These results suggest that hypercalciuria is one of the important metabolic factors contributing to CaOx formation in dogs (Lulich et al., 1991; Furrow et al., 2015; Furrow et al., 2017). To avoid the effect of postprandial calciuresis, the feed was withheld from all dogs at least eight hours. The excessive urinary excretion could have resulted from the elevated filtered load of calcium which might be related to the intestinal hyperabsorption as previously mentioned rather than in dogs (Lulich et al., 1991; Luskin et al., 2019) or decreased renal tubular reabsorption, similar to both hypercalciuric human (Coe et al., 1982) and GHS rats (Frick et al., 2013) failed to reduce urinary calcium excretion under a restriction of dietary calcium intake.



Serum phosphate in CaOx groups was significantly decreased compared to controls, while there was no difference in UP/Cr between these groups. These findings might explain the effect of PTH on renal tubules, which can cause decreased renal reabsorption of phosphate (Prie et al., 2001) or the low P-containing diet (Ohnishi et al., 2014). Although there is no difference in serum magnesium concentration, the UMg/Cr was significantly increased in CaOx dogs which may be a consequence of reduced calcium reabsorption in renal tubules (Martinez et al., 1985) or the effect of PTH (Gill et al., 1967). In addition, magnesium has been known as an inhibitor of CaOx stone formation by reducing calcium and oxalate aggregation which

be effective in acidic urine conditions (Riley et al., 2013). However, the exact mechanism of its effect is less well understood.

Our result showed that 1,25-(OH)2D concentrations were not significantly different between case and control groups, which was similar to previous studies in CaOx stone-forming dogs (Lulich et al., 1991; Groth et al., 2019). This result was also observed in human studies that CaOx nephrolithiasis patients with hypercalciuric condition had either higher or lower serum vitamin D (Kim et al., 2014; Ticinesi et al., 2016). However, we did not measure other vitamin D metabolites to evaluate the vitamin D status. The difference in the 25-(OH)D3/24,25-$(OH)_2$D3 was associated with CaOx stone formation and have been reported in dog (Groth et al., 2019)h and human study (Ketha et al., 2015). These observations could suggest that abnormalities in vitamin D metabolism might be involved in CaOx urolithiasis.

In the present study, we examined the association between VDR polymorphism and the risk of CaOx urolithiasis in dogs. The results showed that the rs852900542 SNP of the *VDR* gene was significantly different between case and control dogs. The presence of the C allele was associated with a 3.82-fold increase in the risk of CaOx urolithiasis. Moreover, the urinary calcium excretion level also correlated with the rs852900542 genotype, in which CaOx dogs with CT or CC genotypes had significantly higher UCa/Cr than those with TT. However, there was no association between rs852900542 genotype and urinary calcium excretion when considering in all dogs from both groups. Therefore, the C allele of the *VDR* gene might be one of the multifactorial factors of CaOx stone formation in dogs.

In our study, the observed allele frequencies of T (0.88) and C (0.12) in control dogs which were not different from the control dogs in another study,

accounted for 0.9 and 0.1 in T and C allele, respectively (Rodriguez-Cortes et al., 2017). While the allele frequencies of T and C in CaOx dogs were 0.69 and 0.31, respectively. This observation suggests that the allele frequency of the rs852900542 SNP varies according to different diseases and study populations. Thus, further studies with larger sample sizes are needed to confirm this result.

The mechanism by which *VDR* gene polymorphism influences the susceptibility to CaOx urolithiasis in dog has not been clarified. However, the previous results in human studies suggest that VDR polymorphisms might influence calcium absorption and excretion. A previous study in Japanese subjects indicated that the T allele of the *TaqI* VDR polymorphism was associated with urinary calcium excretion and the severity of stone formation (Nishijima et al., 2002). The CT of *FokI* polymorphism was associated with stone formation in the Indian population (Bid et al., 2005). In addition, the B allele of the *BsmI* VDR polymorphism was greater in hypercalciuria infants with urolithiasis than in normocalciuric urolithiasis patients in Turkey (Goknar et al., 2016). It should be noted that the *BsmI* and *TaqI* are supposedly non-functional polymorphisms, but they are used as markers for truly functional alleles elsewhere (Uitterlinden et al., 2004). Although the associated SNP in this study is located at the intronic region that may not alter the protein level, the intronic polymorphisms located within 200 base pairs from the nearest splicing site alter the transcription activity or splicing efficiency (Cooper, 2010). Therefore, the intronic SNP may affect the VDR binding affinity or mRNA stability which may influence the vitamin D efficiency (Morrison et al., 1994; Jurutka et al., 2001). As mentioned, the intronic variant might be tagging an associated haplotype, that is, in linkage disequilibrium with the true causal variant. Further studies are needed to test

the association of additional VDR SNPs with CaOx risk with follow-up functional assays to determine the possible impact on gene regulation.

There are some limitations to this study. There were small numbers of dogs enrolled in this study. Moreover, it was difficult to match between control and CaOx urolithiasis dogs. We could not obtain 24-hour urine samples to analyze the correlation between urine calcium excretion and VDR polymorphism because of technical difficulties and inconvenience. Each of control dogs have fed with varied diet, which might affect the excretion of some electrolytes although fasting was required before sample collection to minimize this effect. Another limitation of our study was that some biochemical measurements were not available in all dogs due to inadequate urine and serum samples. Additionally, other factors and hormones that could be involved in CaOx stone formation, including oxalate, citrate, and PTH were not measured in the present study.

**2. Study part II**

In the present study, the breed, age, and sex were matched between groups to reduce other possible factors that could affect urinary protein excretion. All dogs had serum creatinine within normal reference range, which was used to assess kidney function. Dogs with CaOx had lower urine pH compared with stone-free controls. The acidic urine pH does not only affect CaOx crystallization, but also renal tubular cell injury, which can modulate CaOx stone formation. The *in vitro* study of Manissorn and colleagues (2017) revealed that cell death was significantly increased at pH of 5.0. Increased urine specific gravity indicates the concentration of solute in urine which could contribute to the risk of CaOx formation (Mao et al., 2021). However, urine specific gravity was not different between CaOx and stone-free dogs in this study.

All dogs in this study had stones in lower urinary tracts. The protein analysis performed with high-resolution LC-MS/MS led to the identification of 74 urine proteins expressed in study samples. There were forty-nine proteins expressed in both hypercalciuric CaOx and stone-free dogs. The difference in the change of urinary protein might indicate the underlying mechanism that affects the kidney or influence on CaOx formation. Functional analysis of these proteins revealed that many of them are associated with pathways related to blood coagulation, cell adhesion, immunity, and stress response. These findings were consistent with previous proteomic reports. For example, urinary prothrombin fragment 1, was highly abundant in renal stone matrix and linked blood coagulation and urolithiasis (Stapleton et al., 1996). Proteins involved in the blood coagulation such as fibrinogen and vitronectin were identified in both stone matrix and urine from patients with CaOx stone formation (Boonla et

al., 2014). Moreover, CaOx crystals stimulate a release of cytokines and immunoglobulin from human monocytes and in *vitro* study. The high level of immune-response proteins, primarily immunoglobulins was extremely found in nephrolithiatic urine and could be the result of renal tubular damaged caused by CaOx crystals (Wai-Hoe et al., 2009). Adhesion of CaOx crystals on renal tubular epithelial cells can modulate the overproduction of ROS, leading to the production of molecular chaperones such as heat shock protein in response to oxidative stress (Khan, 2014). From the above, the immune response, inflammation ,and coagulation cascade are activated in CaOx urolithiasis in dogs and might be involved in the pathophysiology of the disease.

Our study showed that urinary thrombomodulin was significantly higher in hypercalciuric CaOx dogs compared with stone-free controls. Thrombomodulin is a transmembrane glycoprotein expressed primarily by endothelial cells. Thrombomodulin plays a pivotal role in anticoagulant process by binding of thrombin and activation of protein C resulting in an inhibition of coagulation cascade (Esmon, 2006). Thrombomodulin also has an anti-inflammatory property, including inhibition of leukocyte infiltration. There was a study in children with chronic kidney disease indicated that thrombomodulin was an endothelial dysfunction marker with oxidative stress markers which might contribute to the association of chronic kidney disease (Drozdz et al., 2018). The upregulation of thrombomodulin serves as a glomerular protection process by suppression of glomerular complement activation in diabetic mice (Isermann et al., 2007; Wang et al., 2012). Likewise, thrombomodulin was upregulated in the kidneys of women with pre-eclampsia which might serve as a protective mechanism in response to antiangiogenic stress (van Aanhold et al., 2021).

Inhibition of proinflammatory and profibrotic of thrombin via protease-activated receptors (PARs) pathway is considered as an important protective mechanism of thrombomodulin on the kidney (Kanazawa et al., 2020).

Interestingly, pantetheinase was increased in hypercalciuric CaOx dog. Pantetheinase is one of the hydrolase enzymes which plays a role in the vitamin B5 metabolic process, by hydrolysis of pantetheine to pantothenic acid (vitamin B5) and cysteamine, a potent antioxidant (Kaskow et al., 2012). In addition, this enzyme is encoded by vascular non-inflammatory molecules-1 or vanin-1 (*VNN1*) gene and is expressed mainly in renal tubular epithelial cells, intestine, and liver. Several studies indicate the role of vanin in inflammation and oxidative stress. Increased levels in urinary and serum of vanin-1 were observed in the ethylene glycol-induced kidney injury (Hosohata et al., 2011). Patients with upper urinary tract obstruction (UUTO) which results in acute or chronic kidney disease, had higher urinary vanin-1 value and decreased of vanin-1 value at 4 weeks after intervention (Washino et al., 2019). These results might suggest that vanin-1 is useful for assessing acute kidney injury. In addition, vanin-1 was upregulated in renal ischemia-reperfusion rats, which were affected by oxidative stress (Yoshida et al., 2002). Therefore, the pantetheinase provides tissue with cysteamine via vitamin B5 metabolism in response to oxidative stress injury. However, the study in vanin knockout mice exposed to oxidative stress and had intestinal damage by irradiation revealed that Vanin-$1^{-/-}$ mice was tolerated oxidative stress which could result in an elevated store of glutathione (Berruyer et al., 2004). Thus, vanin-1 promotes inflammation, but could also play a protective role, depending on the affected organ.

Intercellular adhesion molecules-1 (ICAM-1) is a cell surface glycoprotein that belongs to immunoglobulin superfamily molecules that interact with integrins found in leukocytes. ICAM-1 is mainly expressed on endothelial cells of glomerular, peritubular capillaries ,and some interstitial cells. a decrease in ICAM-1 expression was found in glomerulosclerosis, and ICAM-1 levels may be normal or reduced mild nephropathy (Lhotta et al., 1991). Also, the expression of ICAM-1 was reduced in both primary hyperoxaluria type 1 (PH1) patients with kidney stones (KS) or with nephrocalcinosis (NC) compared with PH1 patients without KS and NC (Jayachandran et al., 2020). However, other cell types such as leukocytes and fibroblasts can also express ICAM-1 after exposure to inflammatory stimuli (Piela-Smith et al., 1992). ICAM-1 could be used to indicate inflammation activity in kidney diseases.

The 36 kDa vesicular integral membrane protein (VIP36) is an intracellular lectin of the secretory pathway. VIP36 is not only localized in the Golgi apparatus, but also the plasma membrane, and could be involved in the transport and sorting of glycoproteins (Hara-Kuge et al., 2002). This glycoprotein has been incorporated in human kidney stone matrix (Witzmann et al., 2016; Canela et al., 2020). In addition, another specialized plasma membrane protein called caveolae are a specialized plasma membrane that contains VIP21 protein and others with multiple functions such as several receptor or molecules that participate in signal transduction such as calcium pump, vitamin D receptor, and calcium-sensing receptor (Cao et al., 2003; Cohen et al., 2004). Like the caveolin, other proteins referred to as modifiers of raft function, such as VIP36 could result in changes in raft morphology or biochemical property (Cohen et al., 2004). Many functions related to calcium reabsorption had been reported. Caveolin-1 null mice showed defect in renal calcium reabsorption,

consequently, develop hypercalciuria and urolithiasis (Cao et al., 2003). This could be resulting in loss of membrane protein function in the absence of caveolae.

Accordingly, calcium oxalate crystals react to renal epithelial cells and subsequently cause degenerative changes of renal tubular injury and interstitial fibrosis (Nasr et al., 2008). Based on pathological conditions, kidney stones could be a contributing factor in the AKI, development of CKD ,and its progression (Rule et al., 2009). To date, most studies have widely explored the biomarkers for kidney injury (Alge and Arthur, 2015; Lopez-Giacoman and Madero, 2015; Zhang and Parikh, 2019) which could reflect the pathogenesis and pathobiology of AKI. Unfortunately, these identified urinary proteins may be used as the candidates for urinary tract injury in CaOx urolithiasis in dogs

There are a few limitations to this study. There were small numbers of dogs enrolled in this study. The effect of sex hormone in urine protein excretion could not be ruled out due to there was 1 female dog in each group. The observational cohort study is suggested to evaluate the value of candidate urinary markers and the incidence of CaOx urolithiasis.

# CHAPTER VI

# SUMMARY

## Study part I

In this present study, the rs852900542 *VDR* polymorphism is associated with CaOx susceptibility in dogs, which is related to urinary calcium excretion. This finding suggests that vitamin D metabolism might play a role in CaOx stone risk in dogs. Further investigations are required to identify the role of vitamin D and *VDR* polymorphism to prevent the incidence and recurrence of CaOx urolithiasis in dogs.

## Study part II

In this study, the hypercalciuric CaOx dogs have an increased level of urinary proteins, including thrombomodulin, pantetheinase and VIP36 which indicate that the urinary tract injury. The identification of these urinary proteins may lead to an enhanced understanding of pathophysiology of CaOx urolithiasis in dogs. Further investigations are needed to validate these urinary candidate proteins and to verify their significance in the incidence of CaOx urolithiasis and progression of kidney disease.

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