# Metabolic and Genetic Determinants of Urolithiasis in a Natural Canine Model

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**Abstract**

Calcium oxalate (CaOx) kidney stones are a common health problem across the world. There is a substantial inherited component to CaOx stone risk, but susceptibility genes have largely evaded identification. The goal of this thesis was to use the dog as a natural model to ascertain metabolic and genetic risk factors for urolithiasis.

Cystine and urate stones have previously been reported to have shared susceptibility genes between dogs and people. This thesis demonstrated that 2,8-dihydroxyadenine urolithiasis provides a third example of stone disease with a shared genetic basis between the species. It is likely that there are also overlapping genetic risk factors for CaOx urolithiasis.

Prior to initiating genetic investigations into CaOx urolithiasis, three canine breeds were evaluated for metabolic disturbances associated with stone risk. Each of the breeds was shown to have idiopathic hypercalciuria, the trait underlying stone risk in people. Two breeds were subsequently selected for genome-wide association studies. Three unique susceptibility loci were identified. This supports a polygenic basis for CaOx stone risk in dogs. All three loci harbor plasma membrane transporter genes. One locus on CFA37 was selected for variant discovery using whole genome next-generation sequencing. No putative causal mutations were identified in coding sequence for the positional genes, but markers in and near the top candidate gene, *SLC39A10*, were associated with disease in a large cohort.

*SLC39A10* encodes a plasma membrane metal ion transporter. Several metals have been debated as potential triggers of stone formation. Genotype for the *SLC39A10* risk haplotype was found to correlate with urinary strontium and calcium levels, as well as stone risk. This provides evidence that dogs with the *SLC39A10* risk haplotype may have a functional mutation in the gene that directly or indirectly alters handling of strontium and/or calcium. Though no coding variants were found in *SLC39A10*, the mutation could reside within a regulatory region of the gene. Future sequencing and expression studies are planned to further evaluate *SLC39A10* and genes within the other risk loci for a role in CaOx urolithiasis.

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# CHAPTER 1

## Introduction and literature review

Part I: Nephrolithiasis in humans

Nephrolithiasis (NL) is a significant health problem, with a lifetime incidence of 12% in men and 6% in women (1, 2). Recurrence rates are as high as 15% in the first year and 50% by 10 years (2), and the morbidity caused by kidney stones has a substantial economic effect. NL has the highest patient cost of the top ten emergency department diagnoses (3), and it is estimated that nearly $5 billion are spent annually on the diagnosis and treatment of NL in the United States (4). Importantly, there is increasing evidence that NL is part of a systemic disorder. It has recently been linked to features of metabolic syndrome, including hypertension, obesity, diabetes mellitus, and cardiac disease (5, 6, 7). The incidence of NL is rising in the United States and across the world (8). Despite the global prevalence and importance of this disease, the cellular and molecular pathogenesis of urolith formation remains poorly understood.

Approximately 80% of kidney stones contain calcium, and the majority is composed of calcium oxalate (CaOx) (9). Hypercalciuria (high urinary calcium) is the most common driving force behind CaOx supersaturation, occurring in up to 60% of patients with CaOx NL (10). While hyperparathyroidism can cause hypercalciuria, most NL patients have normal blood calcium and parathyroid hormone levels. This phenomenon was noted as early as 1939 (11) and in the 50's became known as "idiopathic hypercalciuria" (IH) (12). Other less common metabolic disturbances associated with NL risk include hyperoxaluria, hyperuricosuria, and hypocitraturia (9, 13-15).

Strong evidence suggests that genetics play a role in CaOx NL and IH. The first published reports of familial clustering of NL cases occurred in the 1800's (16, 17).

Human kidney stones develop nearly three times more frequently in individuals with a positive family history (18, 19). Heritability ($h^2$) estimates for stone disease range from 46 - 63%, suggesting that at least half of stone risk is genetically determined (18-21). The $h^2$ estimates for IH are similarly high at 40-52% (22, 23).

Several monogenic disorders have been identified that cause hypercalciuria and CaOx stone risk. These include Dent disease, Bartter syndrome, and distal renal tubular acidosis, among others (24-39). Disorders in oxalate metabolism are also well described and cause primary hyperoxaluria and CaOx NL (40-42). See **Table 1** for a list of monogenic disorders associated with CaOx NL. Despite the number of monogenic disorders that have been identified, they are rare conditions that only explain a minority of CaOx stone cases. Additionally, in most of these disorders, NL is part of a greater phenotype that includes serum electrolyte disturbances, renal failure, bone disease, or other organ abnormalities.

Multiple attempts have been made at uncovering susceptibility genes for idiopathic CaOx stones and IH. Family studies support an autosomal dominant mode of inheritance (14, 22, 43), and it has been proposed that the pattern of inheritance can be explained by 3 major loci (14). Linkage studies have identified loci associated with IH and/or CaOx NL, but the numbers of families and individuals included in these studies were small (1-3 and 16-35, respectively), and no specific mutations were reported (44, 45). More recently, genome-wide association studies (GWAS) have been employed using NL cases (all stone types) and stone-free controls. One GWAS located three chromosomal regions associated with NL, but the odds ratios (ORs) were low (1.14 – 1.22) (46). A second GWAS identified synonymous variants in a claudin-family gene

(*CLDN14*) associated with NL (47). *CLDN14* is a compelling candidate gene; its expression is affected by calcium homeostasis (48, 49), and other claudin genes are known to cause rare monogenic forms of IH and CaOx NL (29-31). Yet, the *CLDN14* variants are present in a homozygous state in 62% of the general population and only account for a small portion of the genetic risk for stones (OR = 1.64).

Genetic studies of common diseases are difficult in humans due to great genetic diversity within the population. There is a tendency to locate only common variants with very small effect sizes while missing rare mutations with large effects (50). Even if a gene is responsible for disease risk in most of the population, it will not be easily detected if affected individuals have different mutations within the gene. Each mutation will have a distinct ancestral origin, and they will not be tagged by the same haplotypes. This is often the case even with monogenic diseases in humans. Most of the monogenic NL conditions listed in **Table 1** are caused by a variety of different mutations within the corresponding susceptibility gene.

## Part II: Laboratory animal models of CaOx urolithiasis

Animal models of CaOx urolithiasis are essential to furthering the knowledge of disease pathophysiology. *In vivo* studies have used mice, rats, rabbits, and pigs, yet none of these animals are natural CaOx stone formers. Research on CaOx NL in these species has required induction of stones via diet, lithogenic substances that promote stone formation (the most common of which is ethylene glycol, a.k.a. antifreeze), or genetic modification (51, 52). The use of dietary or pharmaceutical interventions to drive stone formation often promotes hyperoxaluria rather than hypercalciuria, and it results in

fundamentally artificial models of stone formation. These models can be used to investigate factors that contribute to urinary saturation and crystal aggregation, but they provide little information on the natural mechanisms and genetic risk factors behind IH and NL in people. Knockout mouse models of electrolyte transporters (Slc34a1 and Slc9a3r1), an inhibitor of calcium oxalate crystal aggregation (Tamms-Horsfall), and a renal plasma membrane protein (caveolin-1) have been developed, but these genetically modified mice develop renal interstitial calcium phosphate deposits rather than CaOx nephroliths (1, 52). Mice lacking the chloride-oxalate exchanger Slc16a6 develop CaOx cystoliths, but the underlying defect is hyperoxaluria, not IH (53). A reverse situation is observed with the TRPV5 (renal epithelial calcium channel) knockout mouse model. The TRPV5 knockout mice are affected by hypercalciuria, but they do not exhibit renal crystal or stone deposition (1).

Only a single animal model has been developed that both exhibits a genetic state of hypercalciuria and subsequently forms stones, the key features of the human condition. This animal model has been aptly named the genetic hypercalciuric stone-forming (GHS) rat. The GHS rat was developed over 20 years ago through selectively breeding Sprague-Dawley rats with high urinary calcium levels (54). By the 30th generation, members of this genetic line were characterized by hypercalcuria and spontaneous formation of calcium-containing nephroliths. As seen in IH human patients, two main biochemical parameters of calcium homeostasis, parathyroid hormone levels and blood calcium, are normal in GHS rats. At first, the GHS rat seemed like an ideal model to use to identify susceptibility genes for IH and NL. Crosses between GHS and normocalciuric Wistar Kyoto (WKY) rats were used to map IH to a region on chromosome 1 (55). This locus

was confirmed in a subsequent study using congenic rats with the chromosome 1 locus on a normal WKY background (56). However, the IH-associated locus spans more than half of chromosome 1. Over a decade has passed since this locus was identified, and the region has not been narrowed. Novel approaches and alternative models are needed to uncover specific susceptibility genes involved in IH and CaOx NL.

Part III: CaOx urolithiasis in dogs

While most laboratory animals are relatively resistant to CaOx urolith formation, the companion animal population is naturally plagued by the disease. CaOx is the predominant component of canine uroliths, and there are multiple clinical similarities between canine and human CaOx urolithiasis including a peak incidence in the middle-aged, a male predisposition, and high recurrence rates (57-59). Most canine uroliths are found in the lower urinary tract (bladder and urethra) rather than upper urinary tract (kidneys and ureters) as in people, but this could be explained by postural differences between the species. Notably, the key clinical feature of human CaOx NL, IH, is believed to be the major underlying metabolic risk factor for stone formation in dogs as well. More than two decades ago, researchers at the University of Minnesota documented elevated urinary calcium levels in CaOx stone-forming Miniature Schnauzers compared to healthy Beagles, independent of environment and diet (60). Even fasting urinary calcium levels were higher in the Miniature Schnauzers compared to controls. Urinary oxalate excretion was lower in the stone-forming Miniature Schnauzers, and serum metabolic parameters (parathyroid hormone, vitamin D3 levels, calcium) were similar. These features are suggestive of IH, but the use of different breeds for the case and control groups

complicates interpretation of the data. It does not prove that IH underlies stone risk, as the observations could be due to ancestral genetic differences that are independent of disease state. Breed-matched comparisons are needed to determine if the trait of IH segregates within a breed and explains a significant portion of stone risk.

A single canine study on urinary metabolites in CaOx stone-formers has used breed-matched case and control groups (61). The study involved 17 case-control pairs from 14 breeds, and the urinary data was pooled for analysis. Higher urinary calcium and oxalate levels were observed in the case group, but breed-specific results were not reported. Thus, the basic metabolic factors involved in canine CaOx urolithiasis demand further elucidation. If IH can be confirmed as a risk factor for CaOx urolithiasis in one or more breeds, it would strengthen the potential for use of this spontaneous animal model in translational research. Hyperoxaluria in a canine breed would also serve as an important, albeit different, model for urolithiasis.

The principal advantage of the spontaneous canine model is that there is a strong genetic component to CaOx stones in dogs, as observed in people. There is a strikingly high prevalence of CaOx uroliths in certain dog breeds while others appear protected (57, 59). Miniature Schnauzers, Bichons Frise, and Shih Tzus are consistently at the top of the list for calcium oxalate stones, with 10-24 times the risk relative to mixed-breed dogs (59). This observation signifies familial aggregation of disease and supports inherited risk factors.

Part IV: The value of the dog in genetic studies

Studies to determine susceptibility genes for IH and CaOx NL in humans have met with limited success. One of the recent and popular genetic study approaches has been the GWAS. This type of study compares allele frequencies of genetic markers such as single nucleotide polymorphisms (SNPs) between a case and control group. Success relies on the presence of non-random association between one or more of the SNP alleles and the disease-causing mutation, a phenomenon known as linkage disequilibrium (LD). In contrast to people, dog breeds have relatively little genetic diversity. Selective breeding and population bottlenecks has resulted in extensive within-breed LD at 0.5 – 1 Mb, 10-100x that in people (62). This improves the chances that a mutation will be tagged by regional SNPs in a GWAS. A second success-determining factor for a GWAS is limited genetic complexity to the disease risk. The diverse ancestral backgrounds of people complicate the approach. For example, primary hyperoxaluria is caused by at least 3 distinct genes in people, and a variety of different causal mutations are described for each of the genes (63). When multiple disease-causing mutations are present within a case group, it becomes more difficult to identify contributing loci. In this situation, there are different disease-associated SNP alleles for each mutation, and the frequency of each might be too low within the case group to create a strong statistical signal.

Individual dog breeds come from a limited group of ancestors relative to what is observed in people. As a result, within individual dog breeds, disease traits are more often controlled by a small number of mutations with strong effect. These characteristics of extensive LD and a limited number of disease-causing loci strengthen the ability to detect disease loci through genetic studies focused on a single breed (64, 65). In support

of this point, causative mutations for early onset and/or highly penetrant diseases have been identified via GWAS using less than 30 total dogs (66-68), and mutations for adult onset, incompletely penetrant diseases have been located with fewer than 60 dogs (69, 70). For comparison, the human study that identified *CLDN14* as a susceptibility gene for NL used 3,773 cases and 42,510 controls (47).

Not only are dogs well suited for genetic studies, but there are several examples of shared susceptibility genes for urolithiasis between dogs and humans. Specifically, in dogs and humans, urate stones develop from mutations in *SLC2A9* (71-73), and cystine stones form secondary to mutations in *SLC3A1* and *SLC7A9* (74-76). A list of the genetic disorders associated with non-CaOx urolith types is included in **Table 2** (72, 73, 76-81). 2,8-dihydroxyadenine and xanthine stones also occur in dogs (82, 83). The genetic basis of canine 2,8-dihydroxyadenine is reported in **Chapter 2** and the corresponding published article (84), but, to the author's knowledge, the causal genes for xanthine stones have not been evaluated in a canine population.

The overlap in urolithiasis susceptibility genes between humans and dogs is currently only documented for rare monogenic disorders. However, genes that cause simple inherited diseases in dogs can be relevant to complex human counterparts. A classic example comes from narcolepsy. The genetic basis of autosomal recessive forms of the canine disease was discovered over a decade ago via linkage studies with microsatellite markers (85). The gene identified with this approach is called *HCTR2* and encodes the hypocretin receptor 2. At the time, hypocretin was known to be involved in feeding, but its role in the arousal state was not reported. Multiple investigators subsequently discovered that narcoleptic people have loss of hypothalamic neurons that

produce hypocretin, and there is a resultant deficiency of the protein in the brain and cerebrospinal fluid (86). Mutations in the hypocretin gene or receptors are rarely implicated in human narcolepsy (87). Rather, the human disease is believed to be caused by autoimmune destruction of hypocretin-producing neurons. Thus, canine genetic studies have the potential to identify susceptibility genes for CaOx urolithiasis and IH that will provide knowledge about the pathogenesis of the complex human disease.

Part V: Hypotheses and specific aims

**Hypothesis 1 (Chapter 2) –** Dogs with 2,8-DHA urolithiasis have an autosomal recessive mutation in *APRT*, the susceptibility gene for this urolith type in people.

> *Specific Aim 1* **–** Sequence *APRT* in dogs with 2,8-DHA urolithiasis and clinically healthy dogs of similar ancestral lineages to identify a disease-associated mutation.

**Hypothesis 2 (Chapter 3) –** IH underlies CaOx stone risk in dogs, as observed in human stone-formers.

*Specific Aim 2* – Evaluate urinary metabolites (calcium, oxalate, and creatinine) and blood ionized calcium in case and control dogs from high-risk CaOx stone-forming breeds (Miniature Schnauzers, Bichons Frise, and Shih Tzus).

**Hypothesis 3 (Chapters 4 and 5) –** Genetic studies in dogs will identify novel susceptibility genes for CaOx urolithiasis.

*Specific Aim 3a* – Perform case-control GWAS to identify chromosomal loci associated with CaOx urolith formation in the Miniature Schnauzer and Bichon Frise breeds.

*Specific Aim 3b* – Identify a compelling causative mutation for CaOx urolithiasis in dogs by evaluating next-generation sequencing data from the chromosomal loci found in the GWAS.

**Hypothesis 4 (Chapters 6) –** High levels of urinary trace metals, such as Zn and Cd, are associated with CaOx stone risk in dogs.

*Specific Aim 4a* – Test for a relationship between urinary trace metals and CaOx urolithiasis in multiple dog breeds.

*Specific Aim 4b* – Test for a relationship between genotype for a metal ion transporter candidate gene (identified in *Specific Aim 3b*), urinary metals, and IH in Miniature Schnauzer dogs.

| **Table 1. Monogenic disorders associated with CaOx NL in people** | | | | |
|---|---|---|---|---|
| ***Disease*** | ***Gene*** | ***Inheritance*** | ***OMIM*** | ***Reference*** |
| **Associated with hypercalciuria** | | | | |
| Dent disease type I | *CLCN5* | X-linked recessive | 300009 | 24 |
| Dent disease type II | *ORCL1* | X-linked recessive | 300555 | 25 |
| Nephrolithiasis/osteoporosis, hypophosphatemic type I | ***SLC34A1*** | Autosomal dominant | 612286 | 26 |
| Nephrolithiasis/osteoporosis, hypophosphatemic type II | ***SLC9A3R1*** | Autosomal dominant | 612287 | 27 |
| Hyperphosphatemic rickets with hypercalciuria | ***SLC34A3*** | Autosomal recessive | 241530 | 28 |
| Familial hypomagnesemia with hypercalciuria and nephrocalcinosis | *CLDN16* | Autosomal recessive | 248250 | 29, 30 |
| Familial hypomagnesemia with hypercalciuria, nephrocalcinosis, and severe ocular involvement | *CLDN19* | Autosomal recessive | 248190 | 31 |
| Bartter syndrome type I | ***SLC12A1*** | Autosomal recessive | 601678 | 32 |
| Bartter syndrome type II | *KCNJ1* | Autosomal recessive | 241200 | 33 |
| Bartter syndrome type III* | *CLCNKB* | Autosomal recessive | 607364 | 34 |
| Bartter syndrome type V; Autosomal dominant hypocalcemia | *CASR* | Autosomal dominant | 601198; 601199 | 35, 36 |
| Distal renal tubular acidosis | ***SLC4A1*** | Autosomal dominant | 179800 | 37 |
| Distal renal tubular acidosis with preserved hearing | *ATPV0A4* | Autosomal recessive | 602722 | 38 |
| Distal renal tubular acidosis with progressive sensorineural hearing loss | *ATP6V1B1* | Autosomal recessive | 267300 | 39 |
| **Associated with hyperoxaluria** | | | | |
| Primary hyperoxaluria type I | *AGXT* | Autosomal recessive | 259900 | 40 |
| Primary hyperoxaluria type II | *GRHPR* | Autosomal recessive | 260000 | 41 |
| Primary hyperoxaluria type III | *HOGA1* | Autosomal recessive | 613616 | 42 |

Solute carriers are bolded and highlighted in blue. *NL is rare in patients with Bartter syndrome type III.

| **Table 2. Monogenic disorders associated with non-CaOx NL in people** | | | | |
|---|---|---|---|---|
| ***Disease*** | ***Gene*** | ***Inheritance*** | ***OMIM*** | ***Reference*** |
| **Cystine** | | | | |
| Cystinuria type A | ***SLC3A1**** | Autosomal recessive | 220100 | 76 |
| Cystinuria type B | ***SLC7A9**** | Autosomal recessive or dominant | 220100 | 76 |
| **Urate** | | | | |
| Renal hypouricemia type I | ***SLC22A12*** | Autosomal recessive | 220150 | 77 |
| Renal hypouricemia type II | ***SLC2A9**** | Autosomal recessive or dominant | 612076 | 72, 73 |
| Lesch-Nyhan Syndrome; Kelley-Seegmiller Syndrome | *HPRT1* | X-linked recessive | 300322; 300323 | 78 |
| Phosphoribosylpyrophosphate synthetase superactivity | *PRPS1* | X-linked recessive | 300661 | 78 |
| Glycogen storage disease Ia | *G6PC* | Autosomal recessive | 232200 | 78 |
| **2,8-Dihydroxyadenine** | | | | |
| Adenine phosphoribosyltransferase deficiency | *APRT** | Autosomal recessive | 614723 | 79 |
| **Xanthine** | | | | |
| Xanthinuria type I | *XDH* | Autosomal recessive | 278300 | 80 |
| **Orotic Acid** | | | | |
| Orotic aciduria | *UMPS* | Autosomal recessive | 258900 | 81 |

Solute carriers are bolded and highlighted in blue. *Indicates shared susceptibility gene between dogs and people.

# CHAPTER 2

## An *APRT* mutation is strongly associated with and likely causative for 2,8-dihydroxyadenine urolithiasis in dogs

# An APRT mutation is strongly associated with and likely causative for 2,8-dihydroxyadenine urolithiasis in dogs

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**Summary**

2,8-dihydroxyadenine (2,8-DHA) urolithiasis in people is caused by autosomal recessive mutations in the adenine phosphoribosyltransferase gene (*APRT*). 2,8-DHA urolithiasis has recently been reported in two dogs, but, to the authors' knowledge, no studies have yet investigated the genetic basis for susceptibility to the development of 2,8-DHA urolithiasis in this species. Our aim was to sequence *APRT* in dogs affected by 2,8-DHA urolithiasis and compare the results to clinically healthy dogs of similar ancestral lineages. Our hypothesis was that we would identify an autosomal recessive mutation in *APRT* that is associated with the disease. The case population consisted of six dogs with a history of 2,8-DHA urolithiasis: five Native American Indian Dogs (NAIDs) and a mixed breed. The control population consisted of adult NAIDs with no history of urolithiasis. We sequenced *APRT* and identified a missense mutation in a highly conserved codon of *APRT* (c.260G>A; p.Arg87Gln). The c.260A mutation was present in a homozygous state in all six dogs with 2,8-DHA urolithiasis, and it was strongly associated with the disease. This exact missense mutation has been previously reported to cause loss of APRT enzyme function in a human cell line, and it is likely a causative mutation in dogs. Therefore, the dog offers a naturally-occurring genetic animal model for 2,8-DHA urolithiasis.

## Introduction

Adenine phosphoribosyltransferase (APRT) is an enzyme involved in the purine salvage pathway, where it catalyzes the conversion of adenine and 5-phosphoribosyl-1-pyrophosphate to adenosine monophosphate (88). In the absence of APRT, xanthine dehydrogenase (XDH) converts adenine into 2,8-dihydroxyadenine (2,8-DHA), a compound that is highly insoluble in urine. People affected by APRT deficiency (APRTD; OMIM #614723; http://omim.org/entry/614723) experience recurrent 2,8-DHA kidney stones and can develop renal damage secondary to renal crystal accumulation (79, 89). APRTD in humans is an inherited disease caused by autosomal recessive loss-of-function mutations in the adenine phosphoribosyltransferase gene (*APRT*) (79, 88-90).

2,8-DHA urolithiasis has been previously reported in two dogs (82, 91), but to the authors' knowledge, no studies had been performed to determine the genetic basis for the disease in dogs. We performed a search of the Minnesota Urolith Center's records and identified seven dogs with 2,8-DHA urolith submissions. Of these dogs, five were Native American Indian dogs (NAIDs), one was described as an Alaskan Malamute mix, and the final dog was called a "wolf." The NAID is a recently founded breed (**Figure 1**), derived from the Alaskan Malamute, Siberian Husky, German Shepherd Dog, and Chinook; two dogs from Native American Indian reservations were also reportedly included in the founding population for the breed (92). The breed trend for this rare stone type supports an underlying genetic basis and motivated this study.

Several urolith types are known to have shared susceptibility genes between dogs and humans. Examples include urate stones and *SLC2A9* mutations (71, 73), and cystine stones and *SLC3A1* mutations (74, 93). Logically, the top candidate gene for 2,8-DHA

urolithiasis in dogs was *APRT*, the gene implicated in humans with this stone type. Our aim was to sequence *APRT* in dogs affected by 2,8-DHA urolithiasis and compare results to clinically healthy dogs of similar ancestral lineages. Our hypothesis was that dogs with 2,8-DHA urolithiasis share an autosomal recessive mutation in *APRT*.

## Materials and Methods

### *Animals*

A search of the Minnesota Urolith Center database was performed for canine 2,8-DHA uroliths submitted between January 1$^{st}$, 1981 and October 1$^{st}$, 2013. The uroliths were analyzed and confirmed as 2,8-DHA by infrared spectroscopy. Seven canids with this stone type were identified, including five NAIDs, one mixed breed dog (reported to be an Alaskan Malamute mix), and a "wolf." The veterinary clinics that submitted the samples were contacted and asked to request owner participation in the study. Owners for all five of the NAIDs and the mixed breed dog agreed to participate, and DNA samples were obtained from the dogs. Two of the NAIDs were full siblings.

Samples from healthy NAIDs were obtained to serve as a control population with a similar ancestral lineage to the case dogs. Control dogs were required to have no history of urolithiasis or lower urinary tract signs, and they had to be at least 4 years old (selected based on median age of diagnosis of 2,8-DHA urolithiasis in cases). All licensed breeders of NAIDs were contacted to request participation in the study. Two of five NAID breeders agreed to participate. Breeder 1 had not bred any of the case dogs and submitted samples from three control NAIDs of his/her breeding stock. Breeder 2 owned a mating pair that had produced the two sibling case NAIDs. This breeder submitted samples from

his/her entire breeding stock, which included the aforementioned mating pair and six more distantly related control NAIDs. Breeder 2 also contacted owners of full siblings of the two NAIDs from his/her kennel to inform them of the potential problem in the breed and the opportunity to participate in the study. DNA samples from four control NAIDs were submitted from these owners.

DNA samples from dogs of the four breeds (Alaskan Malamute, Siberian Husky, German Shepherd Dog, and Chinook) reported to have served as the foundation of the NAID were also obtained for the purpose of determining the prevalence of any identified mutation(s) in these breeds. These samples came from three sources: 1) dogs owned by faculty, staff, and students at the University of Minnesota Veterinary Medical Center, 2) banked DNA at the Canine Health Information Center (CHIC) database at the University of Missouri, 3) banked DNA at the Canine Genomics Laboratory at the University of Minnesota, and a Chinook breeder. In total, the samples included DNA from 63 Alaskan Malamutes, 58 Siberian Huskies, 61 German Shepherd Dogs, and 3 Chinooks.

Informed consent was obtained from owners of study participants. Samples were obtained directly by owners (cheek swabs), from laboratories (stored DNA), or from veterinarians (blood and tissue) during the course of routine diagnosis and care. Thus, the samples did not fall under a category that requires approval by an Institutional Animal Care and Use Committee.

*APRT sequencing and variant genotyping*

Genomic DNA was extracted from whole blood, cheek swabs, or paraffin-embedded tissue using a commercially available kit.[a] Standard Sanger sequencing of

*APRT* exons was performed for the six cases with 2,8-DHA urolithiasis and 2 NAID controls. Variants identified in all six case dogs and neither control dog were noted. One of these case variants was a putative functional mutation.

NEBcutter[b] was used to identify differences in restriction enzyme sites between the reference sequence of *APRT* and the putative functional mutation identified in the case dogs. An enzyme, Hyp188I, was found to recognize and cut the reference sequence but not the variant. The canine reference sequence was used to design primers to amplify an 848 base pair (bp) product encompassing the putative causal variant. Standard PCR amplification was performed with 30 cycles and a 60°C annealing temperature on a MJ Research PTC-100 thermal cycler.[c] The PCR product was incubated with 5 units of the Hyp188I[d] enzyme at 37°C for 3h.

The PCR-RFLP (restriction fragment length polymorphism) assay products were resolved using gel electrophoresis (**Figure 2**). Dogs homozygous for the reference nucleotide had 362, 310, and 176 bp products. Dogs homozygous for the variant allele had 538 and 310 bp products. Dogs heterozygous for the variant had all 4 products (538, 362, 310 and 176 bp). All of the dogs in the study were tested for the putative causal variant with this PCR-RFLP. Samples from two of the dogs that had been directly sequenced, one case (homozygous for the variant) and one control (homozygous for the reference nucleotide), were used as genotype controls for all assays.

*Statistical analysis*

Two-tailed Fisher's exact tests were used to compare the frequency of the c.260G>A variant and the proportion of dogs with a homozygous genotype for the variant

between case and control groups. A *P* value of <0.05 was considered significant. The age distribution for the dogs failed the Shapiro-Wilk normality test and is therefore reported as median (range). Analyses were performed with an open source, publically available statistical software.[e]

## Results

*Phenotypic description of 2,8-DHA urolithiasis cases and NAID controls*

All six case dogs were neutered males. As described in section 2.1, five dogs were NAIDs and one was a mixed breed. Two of the NAID cases were full siblings. The median age of stone diagnosis was 4 (1 – 10) years. The stones were removed from locations throughout the lower and upper urinary tracts, including the urethra, bladder, ureters, and kidneys. Four of the six dogs had urinary tract obstructions at the time of diagnosis; two had urethral obstructions, one had bilateral ureteral obstructions, and one had both a urethral and a ureteral obstruction. The urolith material was friable and composed of a combination of colors including yellow, green, gray, and black (**Figure 3**). The case with bilateral ureteral obstructions was euthanized for renal failure. In addition to the ureteral obstructions, post-mortem histopathology revealed fibrosing interstitial nephritis, glomerular amyloidosis, and brown crystalline material filling the renal pelvis (**Figure 4**). An additional dog was diagnosed with chronic kidney disease as a young adult, prior to the discovery of 2,8-DHA urolithiasis, but renal histopathology is not available on this dog.

Allopurinol and a low purine diet were recommended for the five cases that were still living. Four of the cases complied with recommendations. Three had no recurrence

of urolithiasis, but the follow-up time was limited (14 months, range 1-28). The fourth dog was noted to have small cystoliths at a recheck ultrasound 8 months after treatment of the original stones. Urine sediment was submitted to the Minnesota Urolith Center and analysis by infrared spectroscopy was consistent with xanthine. The stones were not available to confirm that the composition matched the urine sediment. The fifth case did not follow recommendations and developed recurrence of cystoliths one year after initial diagnosis; the stones were not removed for analysis. After the recurrence, the owner complied with the preventative recommendations, and the dog has not had any further evidence of urolithiasis (20 months follow-up).

The 15 NAIDs in the control group were reported to be healthy at the time of study completion and had no history of urinary disease. All control dogs were at least 4 years of age with a median of 5 (4 – 11) years. Seven of the control dogs were male (five intact and two neutered), and eight were female (six intact and two spayed). As described in section 2.1, the control group included the sire, dam, and four full siblings of the two sibling NAID cases.

*APRT exonic sequencing results*

Exonic sequencing of the *APRT* gene revealed two coding variants present in a homozygous state in all six case dogs. These variants were not found in either NAID control dog. The first variant was in exon 1: c.61G>A resulting in p.Val21Ile. Valine and isoleucine are nonpolar amino acids with similar structures, and comparative analysis with 28 other species demonstrated that isoleucine is a common variant at this codon.

Specifically, it is the reference amino acid for the cow, mouse, and rat. Thus, this neutral missense mutation was not evaluated further.

The second variant was in exon 3: c.260G>A resulting in p.Arg87Gln. Arginine is a basic amino acid, whereas glutamine is an amide, and comparative analysis revealed that arginine is conserved at codon 87 in all 28 other species. This non-conservation mutation was considered a putative functional mutation and selected for genotyping in a larger population of dogs.

*c.260G>A genotyping and association results*

All six cases were homozygous for the c.260A mutant allele, resulting in an allele frequency in this cohort of 100%. In comparison, only 1 of the 15 NAID controls was homozygous for the mutant allele, 7 were heterozygous, and 7 were homozygous for the reference allele. The overall mutant allele frequency in the control group was 30%. Both the A allele and AA genotype were significantly associated with 2,8-DHA urolithiasis ($p = 7 \times 10^{-5}$ and $p = 1 \times 10^{-4}$, respectively). The 63 Alaskan Malamutes, 58 Siberian Huskies, 61 German Shepherd Dogs, and 3 Chinooks all tested homozygous for the reference c.260G allele.

The single NAID control dog that tested homozygous for the c.260A mutation was a 5 year old intact female dog. The breeder of this dog declined stone screening and urine microscopy to evaluate for 2,8-DHA crystals.

## Discussion

This study describes an *APRT* mutation in six dogs with 2,8-DHA urolithiasis. The mutation follows an autosomal recessive mode of inheritance with stone risk only observed in homozygous dogs. This is the first report of a genetic basis for 2,8-DHA urolithiasis in dogs and demonstrates that *APRT* is a shared susceptibility gene for urolithiasis in dogs and humans. Based on review of the literature, the dog is the only naturally-occurring animal model for human APRTD.

APRTD is a rare genetic disorder in people with an estimated prevalence of 1:50,000 – 1:100,000 (88). The most common manifestation of this disease is recurrent 2,8-DHA urolithiasis. Acute kidney injury may occur from urinary tract obstruction by calculi, and chronic kidney disease is a common sequela caused by crystalline nephropathy. The age of onset varies, with many affected individuals remaining asymptomatic until adulthood. The kidney stones and damage that occur as a consequence of APRTD can be prevented by treatment with a XDH inhibitor such as allopurinol or Febuxostat (for patients intolerant of allopurinol). Allopurinol diminishes crystalluria, and urine microscopy can be used in treatment monitoring. Dietary purine restriction and high fluid intake are also recommended. These therapies can not only prevent the consequences of APRTD, but they also help dissolve stones and improve kidney function in patients with renal failure (79, 89).

The clinical manifestation of disease in the 2,8-DHA case dogs in this study was similar to that observed in humans with APRTD. All six dogs were diagnosed with 2,8-DHA stones as adults (at least 1 year of age). Four of the six dogs presented with obstructive disease; two had urethral obstructions, one experienced both urethral and

ureteral obstructions, and one had bilateral ureteral obstructions. Renal histopathology from the dog diagnosed on post-mortem revealed crystalline material in the renal pelvi and chronic renal damage, and a second dog had a history of chronic kidney disease. Allopurinol and a purine restricted diet were recommended for the living dogs. Based on limited follow-up, these therapies may be effective in preventing 2,8-DHA urolithiasis recurrence in dogs with APRTD, as in humans. We do not know the penetrance of the c.260A mutation and lifetime risk for 2,8-DHA urolithiasis in homozygous dogs. However, early initiation of therapy is essential to effective prevention of renal disease in people (79, 89). Therefore, we recommend that even stone-free dogs that test homozygous for the c.260A mutation are started on preventive therapies.

More than 40 mutations in *APRT* have been reported to cause APRTD and 2,8-DHA stone risk in people (88). The type of mutation varies greatly and includes missense, nonsense, indels, and large deletions. Only individuals with two mutant alleles are affected by the disease. Exonic sequencing of *APRT* in dogs with 2,8-DHA stones identified a missense mutation that results in a shift from a basic amino acid (arginine) to an amide amino acid (glutamine) at position 87 of the protein. Review of 28 other available genomes revealed that arginine is a conserved amino acid at this position across all of the species evaluated, including *Caenorhabditis elegans*, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, and multiple plant species. Importantly, a prior study on somatic mutations found that this specific mutation (p.Arg87Gln) occurred in a human cell line and rendered it unable to produce the APRT enzyme (94). The authors of this study also reported a premature translation termination codon (c.259C>G), an in-frame insertion (c.259_260insGAAAGCCCA), and a frame shift insertion

(c.259_260insCCGA) at codon 87 of *APRT* (94, 95). They concluded that codon 87 is a mutational hot spot in human *APRT*. Our finding of a spontaneous canine mutation at this codon suggests that it may be a mutational hot spot in non-human species as well.

We did not identify the *APRT* mutation in any of the reported foundation breeds for the NAID. There are a few possible explanations for this finding. First, the mutation may have arisen in one of the foundation stock for the NAID and therefore be unique to the breed. An argument against this is that one of the six case dogs was reported to be an Alaskan Malamute mix. However, this dog was rescued from a shelter, and the lineage may not be accurate. Given the physical similarities between a Malamute and the NAID, it is possible that the mixed breed dog was a true NAID. Alternatively, the mutation may have come from a foundation breed but be present at too low a frequency to detect with the sample sizes used in the study. We tested 58 - 63 dogs each of the Alaskan Malamute, Siberian Husky, and German Shepherd Dog breeds. When randomly sampling a diploid population, a sample size of 58 is only estimated to capture allele frequencies of at least 5% with a 95% probability (96). For the Chinook, only 3 dogs were available for study participation. With the small sample size for the Chinook breed, even an allele frequency as high as 50% could be missed.

The greatest limitation of this study was the control group. The control dogs were phenotyped based on the absence of clinical signs of urinary tract disease and an age at or above the median for stone diagnosis in cases. Ideally, the controls would have had a urine sample screened for the presence of 2,8-DHA or an APRT enzyme activity test on hemolysates to rule out subclinical cases of APRTD. While urinary 2,8-DHA levels can be evaluated in human patients, veterinary laboratories do not offer screening for this

compound, and we were unable to find a laboratory in the United States to test our canine urine samples. Similarly, the APRT enzyme activity assay is not available or validated in dogs, and we again encountered difficulty identifying a laboratory to test canine samples. Abdominal ultrasonography or contrast studies could have been performed to rule out urolithiasis, but we did not have sufficient funds to cover this testing. Another complicating factor is that half of the control dogs were females compared to none of the case dogs. If the disease penetrance is lower in female dogs, inclusion of this sex in the control group may have resulted in further phenotyping inaccuracies. A single female control dog was homozygous for the variant. This dog is likely an APRTD case, but, as discussed above, phenotyping information was limited. Though we did not have access to assays to measure urinary 2,8-DHA or APRT enzyme activity, we did offer the owner/breeder ultrasonographic screening for sublinical urolithiasis and urine microscopy for 2,8-DHA crystalluria; these tests were declined. Thus, the phenotype of the control dogs may be more appropriately termed "unknown" than "control." Fortunately, inaccuracies in control phenotyping should increase the risk of a type II error (false negative) but not a type I error (false positive). In other words, the association between the mutation and the disease has likely been underestimated due to these limitations.

## Conclusion

We identified an autosomal recessive missense mutation in *APRT* that is strongly associated with canine 2,8-DHA urolithiasis. This mutation alters a highly conserved amino acid and has previously been demonstrated to be a loss-of-function mutation in a human cell line. Thus, the mutation is likely causative for 2,8-DHA urolithiasis risk in

dogs. Dogs with the *APRT* mutation offer a spontaneous genetic animal model for APRTD and 2,8-DHA urolithiasis.

**Footnotes**

[a]Puregene blood core kit, Qiagen Sciences, Germantown, MD

[b]*tools.neb.com/NEBcutter2*

[c] MJ Research, Inc., Watertown, MA

[d]New England BioLabs, Ipswich, MA

[e]R Development Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

**Figure 1.** Photograph of a Native American Indian dog. This breed is reported to be derived from the Alaskan Malamute, Siberian Husky, German Shepherd, and Chinook breeds.

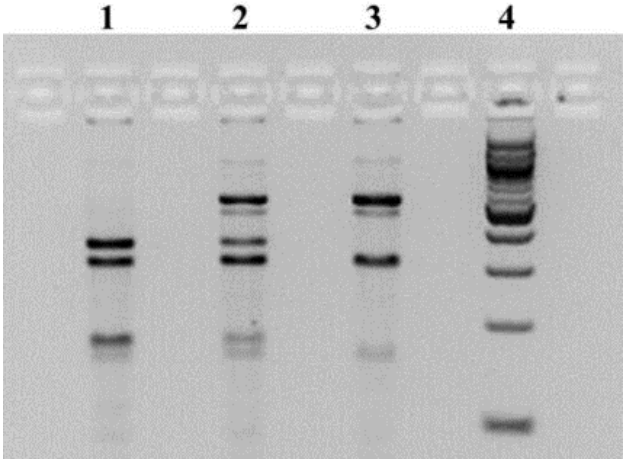


**Figure 2.** *APRT* exon 3 c.260G>A mutation assay using Hyp188I restriction digest of an 848bp PCR product amplified from genomic DNA. Lane 1: Digestion of the reference sequence produces 3 bands (362, 310, and 176 bp). Lane 2: Digestion of sequence from a dog heterozygous for the variant produces 4 bands (538, 362, 310 and 176 bp). Lane 3: Digestion of sequence from a dog homozygous for the variant produces 2 bands (538 and 310 bp). Lane 4: 100 bp ladder.

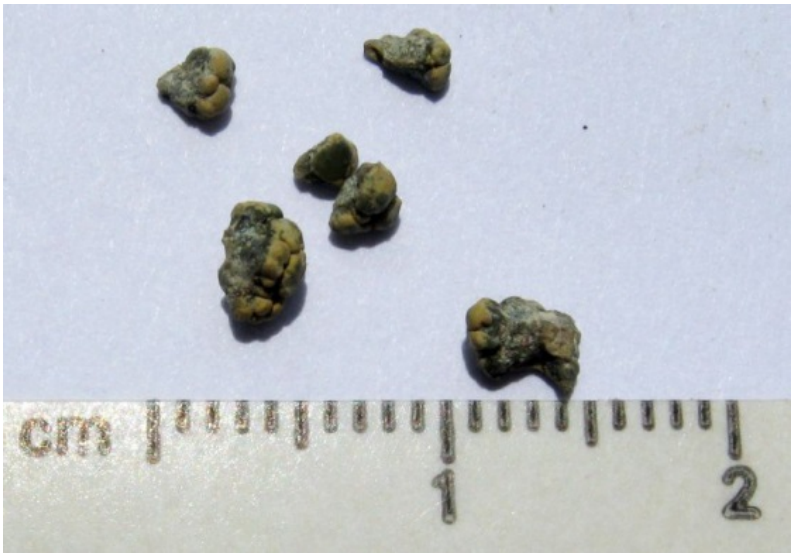


**Figure 3.** Photograph of canine 2,8-DHA uroliths removed from the lower urinary tract.

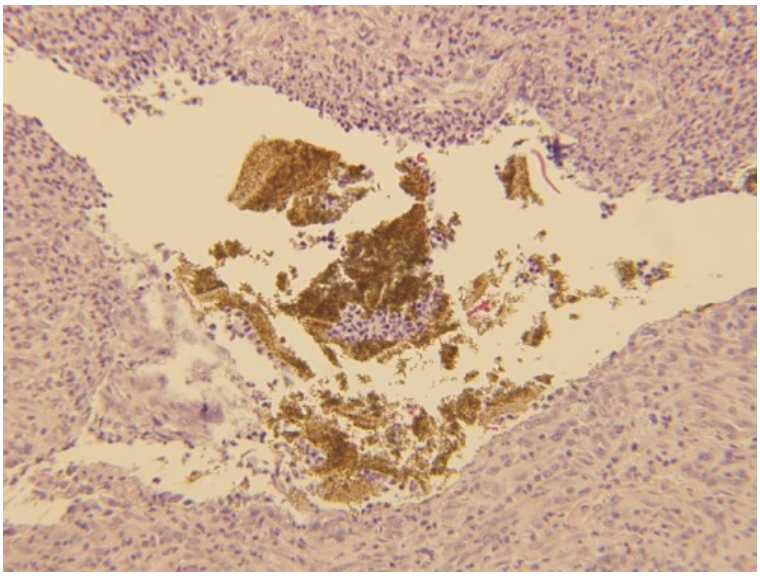

**Figure 4.** Renal histopathology from a dog with renal failure and bilateral ureteral obstructions with 2,8-DHA urolithiasis. Brown crystalline material is observed in the renal pelvis.

# CHAPTER 3

## Fasting urinary calcium-to-creatinine and oxalate-to-creatinine ratios in dogs with calcium oxalate urolithiasis and breed-matched controls

**Fasting urinary calcium-to-creatinine and oxalate-to-creatinine ratios in dogs with calcium oxalate urolithiasis and breed-matched controls**

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## Summary

**Background:** Hypercalciuria and hyperoxaluria are risk factors for calcium oxalate (CaOx) urolithiasis, but breed-specific reports of urinary metabolites and their relationship to stone status are lacking.

**Objective:** Compare urinary metabolites (calcium and oxalate) and blood iCa between CaOx stone-formers and breed-matched stone-free controls for the Miniature Schnauzer, Bichon Frise, and Shih Tzu breeds.

**Animals:** Forty-seven Miniature Schnauzers (23 cases and 24 controls), 27 Bichons Frise (14 cases and 13 controls), and 15 Shih Tzus (7 cases and 8 controls).

**Methods:** Case-control study. Fasting spot urinary calcium-to-creatinine and oxalate-to-creatinine ratios (UCa/Cre and UOx/Cre, respectively) and blood iCa were measured and compared between cases and controls within and across the breeds. Regression models were used to test the effect of patient and environmental factors on these variables.

**Results:** UCa/Cre was higher in cases than controls for each of the 3 breeds. In addition to stone status, being on a therapeutic food designed to prevent CaOx stone recurrence was associated with higher UCa/Cre. UOx/Cre did not differ between cases and controls for any of the breeds. Blood iCa was higher in cases than controls in the Miniature Schnauzer and Bichon Frise breeds and had a moderate correlation with UCa/Cre.

**Conclusions and Clinical Importance:** The trait of hypercalciuria is associated with CaOx stone status in the Miniature Schnauzer, Bichon Frise, and Shih Tzu breeds. Urinary oxalate levels did not correlate with stone status in these 3 breeds. These findings may influence breed-specific stone-prevention recommendations and future genetic study design.

## Introduction

Calcium oxalate (CaOx) urolithiasis is a common urinary tract disease in dogs, but the etiology is poorly understood. In people, hypercalciuria (high urinary calcium) is well established as the most common metabolic abnormality underlying CaOx stone formation (10). Hypercalciuria in the face of normal serum calcium levels is termed idiopathic hypercalciuria (IH). As the name implies, the pathophysiology of IH is largely undefined, but it is believed to be influenced by both environmental and genetic factors. High urinary concentrations of oxalate will also promote CaOx supersaturation, but idiopathic hyperoxaluria (IHO) is a less common metabolic disturbance in human CaOx stone formers (15).

Over the past three decades, several veterinary studies have evaluated urinary calcium and oxalate levels in canine CaOx stone formers (60, 61, 97). The earliest of these studies documented elevated urinary calcium levels in CaOx stone-forming Miniature Schnauzers compared to healthy Beagles, despite controlled housing, diet, and water intake (60). Both fasted and fed 24-hour urinary calcium levels were higher in the stone-forming Miniature Schnauzers compared to Beagle controls. Urinary oxalate excretion was lower in the Miniature Schnauzers, and serum total calcium was not different between groups. These features support the presence of IH in Miniature Schnauzer dogs, but the use of disparate breeds for case and control groups imparts a crucial limitation on the interpretation of this data. Namely, it does not determine if the trait of IH segregates within the breed and associates with stone risk. It is possible that all purebred Miniature Schnauzers have IH, similar to the situation observed with hyperuricosuria in Dalmatians (71).

A subsequent study on urinary metabolites in CaOx urolithiasis used a breed-matched approach (61). Seventeen CaOx stone-forming dogs from 14 different breeds were breed-, age-, and sex-matched to a control group. The stone-former group had both higher 24-hour urinary calcium and oxalate levels relative to the control group. The results of this breed-matched study suggest that IH and IHO are traits that vary within breeds and impart stone risk. However, there were too few dogs of each breed to report breed-specific results. To the authors' knowledge, no studies have been published that report individual dog breed urinary metabolite data for CaOx stone-formers and controls. This information is important for informing breed-based medical recommendations such as hydrochlorothiazide for IH or vitamin B6 for IHO (98).

The first aim of this study was to compare fasting spot urinary calcium and oxalate between CaOx stone-formers and breed-matched controls for each of the top three breeds reported to be at increased risk for CaOx urolithiasis: the Miniature Schnauzer, Bichon Frise, and Shih Tzu. We hypothesized that the stone-formers for each breed would have higher urinary calcium levels than the respective control dogs but that urinary oxalate levels would not differ. A second aim was to compare blood ionized calcium (iCa) between the aforementioned stone-former dogs and breed-matched controls. We hypothesized that iCa would not differ between stone-formers and controls for each of the three breeds.

## Materials and Methods

### *Study Population*

Between February 2011 and March 2014, dogs were recruited from the patient population at the Veterinary Medical Center, University of Minnesota (VMC UMN) and through outreach to primary care veterinary clinics and breed groups in Minnesota and Wisconsin. Purebred dogs of three breeds were recruited: Miniature Schnauzers, Bichons Frise, and Shih Tzus. Cases were defined as dogs with a history of uroliths composed of CaOx (≥70% of the central core), as determined by standard stone analysis at the Minnesota Urolith Center (polarizing light microscopy and infrared spectroscopy). Controls had no history of CaOx uroliths or lower urinary tract disease aside from prior culture-positive bacterial urinary tract infections. To minimize the inclusion of latent stone formers, the control dogs were selected to be ≥ 8 yo (the reported mean age of CaOx urolithiasis diagnosis in dogs) (57). Controls were screened with abdominal radiographs to rule out radio-opaque uroliths and a urinalysis to rule out CaOx crystalluria.

For both groups, dogs were excluded if they had received glucocorticoids within the past week or another drug with known effects on urinary calcium excretion (i.e. furosemide, thiazide diuretics, levothyroxine, theophylline, potassium citrate) within the past 24 hours. Dogs were also excluded if they had a clinical diagnosis of a disease that alters urinary calcium excretion (i.e. hyperparathyroidism, hypercalcemia of malignancy, hyperadrenocorticism, diabetes mellitus, osteolytic disease, granulomatous disease). Dogs in both groups were permitted to be on therapeutic foods designed to minimize CaOx recurrence; dietary data was used in the data analysis. Written informed consent

was obtained from the owners of each study participant, and the study protocol was approved by the University of Minnesota Institutional Animal Care and Use Committee.

### *Laboratory Measurements*

Owners were instructed to withhold food, but not water, for 12-18 hours prior to sample collection. All dogs had voided at least once prior to sample collection to insure that the research urine sample was never the first micturition of the day. Urine was collected by free catch, cystocentesis, or catheterization. Unfiltered 1-3 ml urine aliquots were immediately analyzed for calcium (spectroscopy and the calcium sensitive dye Arsenazo II) and creatinine (modified Jaffe procedure) (99, 100) A 4 ml urine aliquot was filtered through a 0.22 µm polyethersulfone membrane,[a] stored at -80° C, and analyzed for oxalate (ion chromatography on non-acidified urine) within 6 months (101). Spot urinary calcium-to-creatinine and oxalate-to-creatinine ratios in mg/mg (UCa/Cre and UOx/Cre, respectively) were calculated for each dog. Venous blood was collected in a syringe with dry lithium heparin, and iCa and creatinine were determined with a blood gas analyzer.[b]

### *Statistical Analysis*

The distribution of the data was visually inspected with Q-Q plots to determine if transformations or non-parametric tests were necessary. For skewed measurements (the urinary data), Wilcoxon rank sum tests were used to compare UCa/Cre and UOx/Cre between case and control groups for each individual breed and across breeds. Medians were reported for the urinary data. Additionally, this data was log-transformed to achieve

a more normal distribution, and regression analyses were performed on the log-transformed UCa/Cre and UOx/Cre with the following variables as predictors: stone status (case versus breed-matched control), breed, sex (male versus female), age (yrs) as a quadratic, and diet (therapeutic CaOx stone-prevention diet versus other foods). The significance of each predictor was assessed using Type II tests (ANCOVA). The least square means (LSM) and 95% confidence intervals (CI) were calculated and back-transformed to the original scale for reporting. For the normally distributed data (blood iCa and creatinine), Student's t-tests were used to compare values between the case and control groups for each individual breed and across breeds, and data is reported as mean ± standard deviation. A regression analysis was also performed for blood iCa, using the predictors described for the urinary analyses. The Pearson correlation coefficient (r) was calculated to test the relationship between the log-transformed UCa/Cre and blood iCa. All analyses were performed using the R software for statistical computing,[c] and p-value of $<0.05$ was considered significant.

## Results

Forty-seven dogs were recruited as potential cases for the study. Three dogs were excluded due to ionized hypercalcemia identified at the study appointment and subsequent diagnoses of primary hyperparathyroidism (1 Bichon Frise and 1 Shih Tzu) and hypercalcemia of malignancy secondary to an anal gland adenocarcinoma (1 Miniature Schnauzer). Data from the remaining 44 cases, 23 Miniature Schnauzers, 14 Bichons Frise, and 7 Shih Tzus, were included in the analysis. The signalment data for the cases is presented in **Table 1**. More than half (28/44, 64%) of the cases had

experienced recurrent CaOx stones with a median of 2 (0-4) episodes. The mean age at the time of first stone diagnosis was 7.5 ± 2.3 yrs, and the mean age at the time of study enrollment was 9.5 ± 2.3 yrs. Seventeen cases (39%) had cystoliths at the time of sample collection and had their stones removed later that same day.

Sixty-one dogs were screened as potential controls for the study. Sixteen dogs (26%) were excluded because radio-opaque uroliths consistent with a composition of CaOx were detected via screening radiographs. Data from the remaining 45 controls (24 Miniature Schnauzers, 13 Bichons Frise, and 8 Shih Tzus) were included in the analysis. The mean age of the control dogs at the time of study enrollment was 10.5 ± 1.9 yrs.

Within each breed, age at the time of study enrollment and sex were not significantly different between cases and breed-matched controls (**Table 1**). However, for across breed comparisons, the age of the entire control group was older than the case group ($p = 0.03$), and more cases than controls were male ($p = 0.03$). Fourteen cases and one control were fed therapeutic foods designed to prevent CaOx recurrence;[d,e] the control dog was fed the therapeutic food[e] because a household dog had been affected with CaOx stones.

UCa/Cre was significantly higher in cases than controls for each of the three breeds (**Table 1**, **Figure 1A**) and across breeds ($p < 0.001$). In the UCa/Cre regression analysis, stone status was a strong predictor of UCa/Cr ($p < 0.001$, **Table 2**). Being on a therapeutic food for CaOx stone-prevention also had a positive correlation with UCa/Cre ratios ($p = 0.03$). There was no significant effect of breed, sex, or age on UCa/Cre. UOx/Cre was not different between case and controls for any of the three breeds (**Table**

**1**, **Figure 1B**), and none of the predictors in the UOx/Cre regression analysis were significant (**Table 2**).

Blood iCa was within the reference range (5.1-5.9 mg/dL) for all but one dog: a Bichon Frise control with a low calcium at 4.9 mg/dL. Blood iCa was significantly higher in cases than controls for the Miniature Schnauzer and Bichon Frise breeds (**Table 1**, **Figure 1C**) and for across breed comparisons between cases and controls ($p < 0.001$). Stone status was the only significant predictor of blood iCa in the regression model (**Table 2**). There was a moderate positive correlation between blood iCa and UCa/Cre ($r = 0.31$, $p = 0.007$). Blood creatinine did not differ between cases and controls within or across breeds.

## Discussion

This study demonstrates that fasting spot UCa/Cre ratios are higher in dogs with CaOx uroliths than breed-matched controls for the Miniature Schnauzer, Bichon Frise, and Shih Tzu. These results support our hypothesis that hypercalciuria is an important underlying metabolic abnormality associated with stone formation in these 3 dog breeds. UOx/Cre ratios did not associate with stone status for any of the breeds studied. An unexpected finding was higher blood iCa levels in stone-formers than controls even though no values were above the reference range.

In people, hypercalciuria is the most common driving force behind CaOx supersaturation, occurring in up to 60% of patients with kidney stones (10). Most hypercalciuric patients have normal blood calcium, and the phenomenon has been termed “idiopathic hypercalciuria” (IH) (12). None of the dogs included in this study were

hypercalcemic. Thus, the hypercalciuria for the 3 breeds is appropriately deemed IH. Three disturbances in calcium homeostasis are theorized to contribute to IH in stone-formers: intestinal hyperabsorption, excessive bone resorption, and decreased renal resorption of calcium. A prior study on urinary metabolites in Miniature Schnauzers with CaOx urolithiasis proposed intestinal hyperabsorption as the likely mechanism behind IH in the breed (60). The authors of that study based their theory on the finding of more pronounced hypercalciuria in fed versus fasted states. The prior study did not detect differences in total serum calcium between stone-formers and controls, but total calcium has a 27% diagnostic discordance for predicting iCa status (102). In the present study, the stone-formers were shown to have significantly higher blood iCa levels than the control group. This finding supports either intestinal hyperabsorption or bone resorption as the cause. However, a concurrent defect in renal calcium resorption cannot be ruled out. Human studies have shown that multiple disturbances can be present simultaneously (10), and research on urinary calcium excretion relative to net calcium absorption and bone mineral loss would be needed to determine the source of IH in dogs. Furthermore, though the prior study on stone-forming Miniature Schnauzers found vitamin D and parathyroid hormone levels to be normal (60), these hormones were not measured in this study.

In the previous Miniature Schnauzer study (60) and another on urinary metabolites in dogs and cats with CaOx urolithiasis (97), urinary calcium was higher, but urinary oxalate was lower in stone-formers compared to controls. In contrast, another report on urinary metabolites in CaOx stone-formers found both higher urinary calcium and oxalate levels in a group of stone-forming dogs relative to a breed-matched control group (61). This study did not detect differences in UOx/Cre between stone-formers and

controls for any of the individual dog breeds or when data was analyzed across breeds. The conflicting results between studies may be due to evaluation of different dog breeds. The report that found higher urinary oxalate included 14 diverse breeds. Results for each individual breed were not reported, and one or more may have been affected by IHO instead of or in addition to IH. IHO is reported in approximately 30% of human kidney stone patients (15). Hypocitraturia is also associated with human stone risk (13, 15); urinary citrate was not measured in this study.

In people, urinary metabolite measurements are often used to guide treatment decisions and monitor response to preventative measures (98). This study successfully used fasting spot UCa/Cre and Ox/Cre ratios to compare groups of CaOx stone-formers to breed-matched controls, but it remains to be determined as to whether these measurements can be used to characterize individual dogs. Though the stone-formers had significantly higher spot UCa/Cre ratios than controls, there was great variability between dogs, and overlap was present between the case and control groups for all 3 breeds. There are a few possible explanations for this finding. First, 24-hour urinary collection is a superior method of screening for hypercalciuria than fasting spot UCa/Cre in people. Human studies have reported varying correlations ($r = 0.52 – 0.76$) between these two techniques, and fasting spot UCa/Cre may systemically underestimate urinary calcium (103-106). Therefore, the spot measurements used in this study may have missed mild cases of hypercalciuria. Measurement of 24-hour urinary calcium excretion would have been preferable, but this technique was not used due to the expense, owner inconvenience, and technical difficulty of complete urine collection in dogs. Second, even within a breed, there could be multiple risk factors present for the development of

CaOx urolithiasis. As stated earlier, we did not measure urinary citrate or molecular inhibitors of stone formation. A third explanation for the overlap between spot UCa/Cre in cases and controls is that some cases may have experienced hypercalciuria at the time of stone formation from a temporary environmental or drug exposure that resolved before the time of study participation. Finally, there were a couple high UCa/Cre outliers in the control groups. These control dogs with IH may not have formed stones due to a protective environmental or genetic factor, or they could have had small stones that were either passed or not detected by survey radiography.

The results of this study have important implications for genetic study design. Miniature Schnauzers, Bichons Frise, and Shih Tzus are consistently reported to have a significantly increased risk for CaOx urolithiasis relative to mixed breed dogs (57, 59). Strong breed predispositions signify familial aggregation and strongly support the presence of inherited risk factors for CaOx stones. In order to design a study to identify genetic risk factors for CaOx uroliths in dogs, it is essential to understand whether the trait of interest segregates within the breed or is a fixed trait. Traits that segregate within a breed can be investigated with a breed-matched case-control approach. In contrast, traits that affect the entire breed require a different approach such as mapping with interbreed backcrosses, comparison of affected breeds to protected breeds, or homozygosity mapping. The results of this study demonstrate that hypercalcuria is not a fixed trait in the Miniature Schnauzer, Bichon Frise, or Shih Tzu breed, and a breed-matched case-control genome-wide association study is currently underway to identify susceptibility genes for canine CaOx urolithiasis.

An unexpected finding in this study was the strikingly high incidental stone prevalence in the population screened as potential controls. More than a quarter of the dogs recruited as controls were identified to have radio-opaque cystoliths and/or nephroliths on radiographs. The owners of these dogs did not report any clinical signs of lower urinary tract disease, despite some dogs having a large stone burden. The true prevalence of stones in these 3 breeds cannot be determined with this data, but it is likely much higher than the estimated all breed prevalence of 0.3-2% (107).

In people, there is a documented relationship between nephrolithiasis and increased risk for chronic kidney disease (108). There was no association between stone status and blood creatinine within the individual breeds or across breeds in the present study. The absence of a link could be due to stone type (risk in people may be specific to struvite and urate stones), location (cystoliths versus nephroliths), or the imprecision of using creatinine to estimate renal function.

A limitation of this study is that urinary oxalate and/or blood measurements (iCa and creatinine) were not available for a subset of the dogs. This was due to inadequate urine volume in the case of urinary oxalate and either blood clotting or absence of appropriate sample collection in the case of the blood measurements. Without iCa on all dogs, we cannot rule out that some cases may have had hypercalcemia. Additionally, though we excluded dogs with a clinical diagnosis of a disease that alters urinary calcium excretion, it was not feasible to screen each case and control dog for all underlying diseases that could influence urinary calcium.

Another limitation is that the case and control dogs were not matched on patient characteristics such as age and sex. In people, urinary calcium peaks in middle age (109)

and is higher in males than females (110). Thus, matching of these characteristics would have been ideal. It was not performed due to the age limitation required for controls (older dogs were recruited to minimize the risk of enrolling latent cases) and the difficulty encountered recruiting a sufficient numbers of male controls (many were excluded due to incidental stones on screening). Despite these obstacles, age (analyzed as a quadratic to permit a curved relationship) and sex for case and breed-matched control groups were not different within each individual breed. When the breed data was combined, the case group was significantly younger overall and had a higher proportion of males than the control group, but neither age nor sex affected UCa/Cre or UOx/Cre in the regression models.

Diet was also not controlled or matched between cases and controls. A standardized diet would have added a level of inconvenience and expense and was not possible for this study. A 12-18 hour fast was required prior to sample collection to help minimize the effects of variation in nutrient intake on urinary metabolites, but this would not be expected to eliminate all effects of diet. Furthermore, 15/89 dogs (14 cases and 1 control) were on therapeutic foods that are specially formulated to reduce the precipitation of CaOx. Interestingly, being on such a diet was associated with an increase in UCa/Cre in the regression model. This does not mean that the therapeutic diets directly increase urinary calcium, especially given published data to the contrary (111). Rather, the difference could be explained by stricter diet recommendations for the more severe stone-formers suffering from higher urinary calcium levels and greater stone recurrence than those permitted to eat an over-the-counter diet. There were insufficient dogs on the therapeutic CaOx stone-prevention foods to investigate this theory.

In conclusion, IH is a common metabolic abnormality in Miniature Schnauzer, Bichon Frise, and Shih Tzu dogs with a history of CaOx urolithiasis. Due to overlap between case and control groups and undetermined intra-individual variability, it is unknown if UCa/Cre ratios will be useful in the clinical monitoring of individual dogs. However, in general, stone-formers of these breeds may benefit from preventative treatments directed at lowering urinary calcium, such thiazide diuretics (98, 111).

**Footnotes**

[a]Millex®GP Filter Unit, Merck Millipor Ltd, Cork, IRL

[b]i-STAT 1, Abbott Point of Care Inc, East Windsor, NJ

[c]R Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/

[d]Prescription Diet canine u/d, Hill's Pet Nutrition Inc, Topeka, KS

[e]Royal Canin Veterinary Diet canine urinary SO, Waltham Centre for Pet Nutrition, Leicestershire, UK

**Table 1.** Patient characteristics, median fasting spot urinary calcium-to-creatinine and oxalate-to-creatinine ratios (UCa/Cre and UOx/Cre, respectively), and mean blood ionized calcium and creatinine in dogs with a history of calcium oxalate stones (cases) and breed-matched stone-free dogs (controls).

| Breed | Age (yrs) | Sex (M/F) | UCa/Cre (mg/mg) | UOx/Cre (mg/mg) | iCa (mg/dL) | Creatinine (mg/dL) |
|---|---|---|---|---|---|---|
| **Miniature Schnauzer** | | | | | | |
| Cases, 23 | 9.1 ± 2.4 | 19/4 | 0.067*** | 0.034 (19) | 5.5** (20) | 0.9 (20) |
| Controls, 24 | 10 ± 1.4 | 15/9 | 0.022 | 0.033 (20) | 5.3 (23) | 0.9 (23) |
| **Bichon Frise** | | | | | | |
| Cases, 14 | 10.1 ± 2.3 | 11/3 | 0.093** | 0.068 (11) | 5.5* (10) | 0.8 (10) |
| Controls, 13 | 10.9 ± 2.4 | 8/5 | 0.041 | 0.076 | 5.3 (11) | 0.8 (11) |
| **Shih Tzu** | | | | | | |
| Cases, 7 | 9.1 ± 2.1 | 5/2 | 0.12*** | 0.079 | 5.6 (5) | 0.8 (5) |
| Controls, 8 | 11.1 ± 2.2 | 3/5 | 0.028 | 0.038 (7) | 5.4 | 0.8 |

UCa/Cre and UOx/Cre were obtained by dividing urinary calcium or oxalate, respectively, in mg/dL by urinary creatinine in mg/dL. For measurements that were not available for all dogs, the total number of dogs with the measurement is listed in parentheses. Significantly higher values in cases compared to controls (within-breed comparisons) are denoted with * ($p < 0.05$), **($p < 0.01$) or *** ($p < 0.001$).

**Table 2.** Analysis of covariance (ANCOVA) results for the effects of patient and environmental factors on urinary calcium-to-creatinine (UCa/Cre) and oxalate-to-creatinine (UOx/Cre) ratios and blood iCa. UCa/Cre and UOx/Cre were log-transformed for the regression and then back-transformed such that the least square means (LSM) and 95% confidence intervals (CI) are reported in the original scale.

| | LSM | 95% CI | D.f. | F value | P-value |
|---|---|---|---|---|---|
| **logUCa/Cre** | | | | | |
| **Stone status** | | | 1 | 34 | **< 0.001** |
| Case | 0.041 | 0.026 – 0.063 | | | |
| Control | 0.009 | 0.005 – 0.014 | | | |
| **Breed** | | | 2 | 0.3 | 0.7 |
| Miniature Schnauzer | 0.017 | 0.010 – 0.026 | | | |
| Bichon Frise | 0.020 | 0.012 – 0.033 | | | |
| Shih Tzu | 0.021 | 0.011 – 0.039 | | | |
| **Sex** | | | 1 | 2.7 | 0.1 |
| Male | 0.015 | 0.010 – 0.023 | | | |
| Female | 0.023 | 0.014 – 0.039 | | | |
| **Age** | NA | NA | 2 | 1.8 | 0.2 |
| **Diet** | | | 1 | 5.1 | **0.03** |
| CaOx prevention diet | 0.028 | 0.015 – 0.054 | | | |
| Other | 0.013 | 0.009 – 0.018 | | | |
| **logUOx/Cre** | | | | | |
| **Stone status** | | | 1 | 0.25 | 0.6 |
| Case | 0.009 | 0.005 – 0.017 | | | |
| Control | 0.011 | 0.006 – 0.022 | | | |
| **Breed** | | | 2 | 2.4 | 0.1 |
| Miniature Schnauzer | 0.009 | 0.004 – 0.017 | | | |
| Bichon Frise | 0.018 | 0.009 – 0.036 | | | |
| Shih Tzu | 0.007 | 0.003 – 0.016 | | | |
| **Sex** | | | 1 | 0.39 | 0.5 |
| Male | 0.011 | 0.006 – 0.023 | | | |
| Female | 0.009 | 0.005 – 0.016 | | | |
| **Age** | NA | NA | 2 | 2.5 | 0.09 |
| **Diet** | | | 1 | 0.38 | 0.5 |
| CaOx prevention diet | 0.009 | 0.005 – 0.014 | | | |
| Other | 0.012 | 0.005 – 0.030 | | | |
| **iCa** | | | | | |
| **Stone status** | | | 1 | 8.6 | **0.005** |
| Case | 5.5 | 5.4 – 5.6 | | | |
| Control | 5.3 | 5.2 – 5.4 | | | |
| **Breed** | | | 2 | 1.5 | 0.2 |
| Miniature Schnauzer | 5.4 | 5.3 – 5.5 | | | |
| Bichon Frise | 5.3 | 5.2 – 5.4 | | | |
| Shih Tzu | 5.5 | 5.3 – 5.6 | | | |
| **Sex** | | | 1 | 3.5 | 0.07 |
| Male | 5.4 | 5.3 – 5.5 | | | |
| Female | 5.3 | 5.2 – 5.4 | | | |
| **Age** | NA | NA | 2 | 0.2 | 0.8 |
| **Diet** | | | 1 | 0.5 | 0.5 |
| CaOx prevention diet | 5.4 | 5.3 – 5.5 | | | |
| Other | 5.4 | 5.2 – 5.5 | | | |

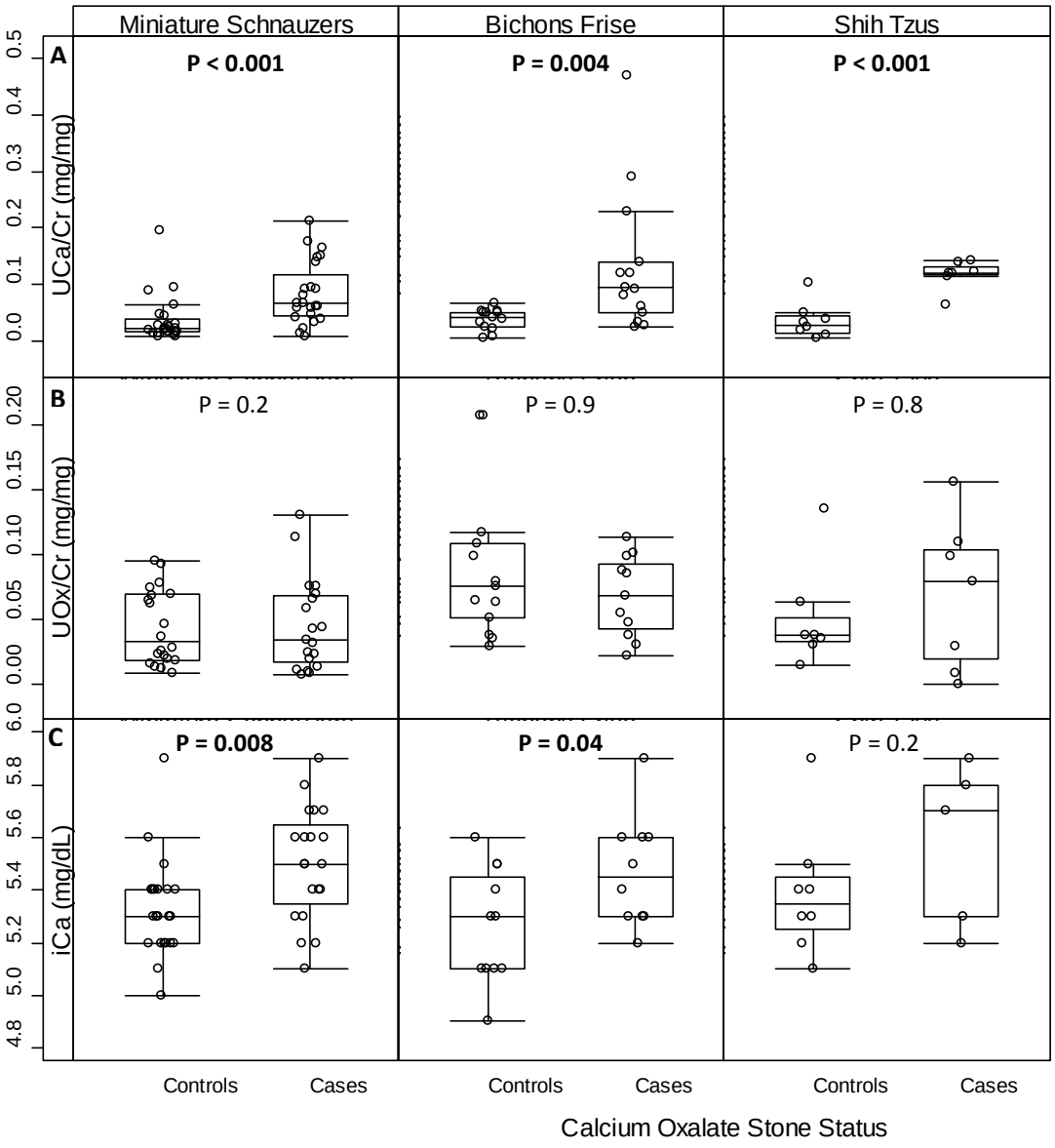


**Figure 1.** Box and whisker plots of fasting spot urinary **A**) calcium-to-creatinine (UCa/Cre, mg/mg) and **B**) oxalate-to-creatinine (UOx/Cre, mg/mg) ratios and **C**) blood iCa (mg/dL) for dogs with a history of calcium oxalate stones (cases) and breed-matched stone-free dogs (controls). The boxes represent the interquartile range ($25^{th}$ – $75^{th}$ percentile), the horizontal line within the boxes represents the median, and the whisker bars extend to 1.5 times the interquartile range. Dots represent individual dog measurements; those that fall above or below the whisker bars are outliers.

# CHAPTER 4

## Genome-wide association study of CaOx urolithiasis in two dog breeds

**Genome-wide association study of CaOx urolithiasis in two dog breeds**

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**Summary**

Calcium oxalate (CaOx) is the most common type of kidney stone in people, affecting roughly 10% of the population. We used a spontaneous canine model of the disease to investigate genetic risk factors for CaOx urolithiasis. Case-control genome-wide association studies were performed in the Miniature Schnauzer and Bichon Frise. Three susceptibility loci were identified: 1) CFA37 g.7061552 – 10653612 bp, 2) CFA15 g.32320032 – 33584438 bp, and 3) CFA15 g.42879417 – 47864748 bp (locations are based on the CanFam2.0 assembly). One of the CFA15 regions (g.42879417 – 47864748 bp) was independently associated with stone risk in both breeds and contained SNPs that achieved genome-wide significance in a combined breed mixed model analysis with correction for population stratification ($p_{raw}$ = 1.6 x 10-7, $p_{genome}$ = 0.004). The other two loci were only associated with stone risk in the Miniature Schnauzer breed. These findings suggest that CaOx urolithiasis is a polygenic disease in dogs, and multiple genetic risk factors may be present even within a single breed. All three of the susceptibility loci identified in this study harbor plasma membrane transporter genes, two of which are solute carriers, the most common gene class implicated in hereditary stone disease. Validation of these risk loci in independent populations and sequencing of the positional transporter genes are indicated.

## Introduction

Kidney stones are a common and painful condition. The lifetime risk is 12% for men and 6% for women, and 50% of newly diagnosed patients experience recurrence within 10 years (1, 2). The majority of kidney stones are composed of calcium oxalate (CaOx), and idiopathic hypercalciuria (IH) is the most common abnormality underlying stone risk (9). While diet and environment are influencing factors, more than 50% of the risk for CaOx urolithiasis is estimated to be genetically determined (20). However, the genetic complexity and poorly understood pathogenesis of the disease has made it difficult to elucidate genetic risk factors. Rare monogenic stone disorders have been described, but they account for risk in the minority (<5%) of kidney stone formers (112). A genome-wide association study (GWAS) identified variants in a claudin-family gene (*CLDN14*) that are associated with kidney stone risk (47). The variants are highly prevalent in the general population (62% of people are homozygous), and they only explain part of the genetic risk for stones (OR = 1.64). Other genes with equal or greater effect likely exist.

Dogs offer a naturally occurring animal model of CaOx urolithiasis. As with humans, canine CaOx uroliths are common, heritable, and associated with IH. Dogs and humans share susceptibility genes for several urolith types including urate, cystine, and 2,8-dihydroxyadenine (71-76, 79, 84/**Chapter 2**). Thus, research on the genetic basis of canine CaOx urolithiasis has the potential to identify novel genetic risk factors for the disease in people.

We performed a genome-wide association study (GWAS) to identify susceptibility genes for CaOx urolithiasis in a spontaneous canine model. The Miniature Schnauzer and Bichon Frise breeds were selected for the study due to their high reported risks for CaOx stones (OR = 22- 24 relative to mixed breed dogs) (59). Single and combined breed approaches were used. We hypothesized that we would uncover one or more loci associated with CaOx urolithiasis risk in dogs.

## Materials and Methods

*Ethics statement and sample collection*

The study protocol (including sample collection and phenotype characterization procedures) was approved by the University of Minnesota Institutional Animal Care and Use Committee. The study participants were pet dogs, and written informed consent was obtained from owners. Whole blood (3-4 ml in an EDTA tube) was collected from each dog. DNA extraction was performed using Qiagen kits.[a] Extracted DNA was stored at -20 C for up to 5 years prior to SNP genotyping.

*Phenotype characterization*

Purebred Miniature Schnauzer and Bichon Frise dogs were enrolled in the study. Cases were classified as confirmed or presumptive based on the strength of the phenotype. Confirmed cases were dogs diagnosed with 100% CaOx uroliths, as determined by standard stone analysis at the Minnesota Urolith Center (polarizing light microscopy and infrared spectroscopy). Presumptive cases included dogs with mixed

composition stones (≥25% CaOx), as well as dogs with uroliths identified on imaging that were not analyzed but believed to be CaOx based on radiographic appearance and urinalysis results. Controls had no history of CaOx uroliths and were screened with abdominal radiographs to rule out radio-opaque uroliths and a urinalysis to rule out CaOx crystalluria. For those with available fasting urinary measurements (see **Chapter 3**), control dogs with a UCa/Cr greater than 1.5 times the interquartile range for the control group (i.e. outliers) were excluded. The case and control age exclusions varied between GWAS analyses and are described below. Where available, pedigrees were reviewed and used to minimize selection of closely related individuals.

*Miniature Schnauzer GWAS I (initial population)*

One hundred three Miniature Schnauzers were selected for initial SNP genotyping (GWAS I). Cases were ≤ 10 yo and controls were ≥ 9 yo at the time of stone diagnosis or screening, respectively. Sixty-two cases and 41 controls were included in GWAS I.

*Miniature Schnauzer GWAS II (expanded population)*

An additional cohort of 17 cases (16 confirmed and 1 presumptive) and 6 control Miniature Schnauzers were subsequently genotyped for a total of 126 dogs in GWAS II.

*Miniature Schnauzer GWAS III (refined population), Bichon Frise GWAS, and Multibreed GWAS*

Stricter phenotyping criteria were used for these three analyses. Only confirmed cases were included, and all cases were ≤ 8 yo (the median age of stone diagnosis). The control age criterion was also altered to only include controls ≥ 10 yo. The new control age cut-off was selected to be above the 75$^{th}$ percentile for age at stone diagnosis for the cases. Ninety-two of the Miniature Schnauzers (49 cases and 43 controls) from GWAS II met the stricter criteria and were included in GWAS III. The Bichon Frise GWAS cohort comprised 26 purebred Bichon Frise dogs (16 cases and 10 controls). The multibreed GWAS included the 92 Miniature Schnauzers from GWAS III and the 26 Bichons Frise for a total of 118 dogs (65 cases and 53 controls).

*Single nucleotide polymorphism (SNP) genotyping and analysis*

SNP genotyping was performed with the Illumina CanineHD BeadChip (173,662 SNPs, bp locations based on CanFam 2.0).[b] PLINK, a whole genome analysis toolset, was used for analysis of the genotype results (113). Standard data quality measures were taken, including exclusion of individuals with call rates <90%, SNPs with genotyping calls <90%, SNPs with a minor allele frequency <5%, SNPs with differential missingness between cases and controls at $p \leq 0.01$, and SNPs that reject the hypothesis of Hardy-Weinberg equilibrium in the control population at $p \leq 0.001$.

Due to the male predisposition for CaOx uroliths noted in clinical practice (59) and in the study population, sex was considered as a covariate for all analyses. Three

inheritance models were tested for the individual breed GWAS analyses: additive, dominant, and recessive. To obtain a p-value for genome-wide significance, 1,000 label-swapping permutations were performed for each analysis to calculate an empirical p-value within $1/1{,}000^{th}$ of a decimal place (114). For the multibreed GWAS, the Cochran-Mantel-Haenszel (CMH) test was used to account for breed, and label-swapping permutations were conducted within breed clusters. An additional analysis was performed on the Miniature Schnauzer GWAS III population using the top disease-associated haplotype identified in the multibreed GWAS as a covariate along with sex.

To evaluate for population stratification, the genomic inflation factor (GIF) for each analysis was calculated from the p-value distribution for each regression analysis ($GIF_{reg}$) and for a chi-square test ($GIF_{chi}$). For analyses where either $GIF_{reg}$ or $GIF_{chi}$ was > 1.10, a mixed model approach was performed with the GenABEL software (115). In addition to sex, the first principal component was considered as a covariate in the mixed models to provide further correction for population structure.

*Haplotype analysis*

For each GWAS that produced significant results in individual breeds, the SNP genotypes within a 10 Mb region (5 Mb on each side) encompassing the genome-wide significant SNPs ($p < 0.05$) were used for haplotype analysis. For the multibreed GWAS, a 20 Mb region was selected to cover the top 10 SNPs. The Gabriel *et al* approach was implemented with Haploview software to partition the region into haplotype blocks with strong linkage-disequilibrium (116, 117). Haplotypes were also inferred from the

genotype data with the fastPHASE program and visually examined (118). Chi-square tests were performed to test the association of haplotype blocks with disease, and odds ratios (OR) are reported. The regions encompassing the top 5 risk haplotype blocks are reported for the individual breed analyses; the top 10 risk haplotype blocks are reported for the multibreed analysis due to the presence of two separate susceptibility loci within the 20 Mb region. All bp locations are based off the CanFam 2.0 assembly. However, protein-coding genes within the top haplotype regions were determined using the updated annotation from the CanFam 3.1 assembly (119). In the multibreed analysis, the top haplotype for each risk locus was tested for disease association using the CMH test in addition to the chi-square tests.

## Results

*Miniature Schnauzer GWAS I (initial population)*

Miniature Schnauzer GWAS I included 62 cases with CaOx urolithiasis (≤ 10 yo, 52 confirmed and 10 presumptive) and 41 stone-free controls (≥ 9 yo). Association analysis revealed a strong signal on CFA37 for all three inheritance models (**Table 1, Figures 1-3**). The association reached genome-wide significance for several SNPs in the additive model (top SNP = BICF2P650021, g.10603162 bp, $p_{raw} = 3.6 \times 10^{-6}$, $p_{genome} = 0.001$) and for a single SNP in the recessive model (same SNP, $p_{raw} = 1.9 \times 10^{-5}$, $p_{genome} = 0.007$). Due to a $GIF_{chi}$ of 1.13 in the additive model (**Figure 4**), a mixed model was performed with sex and the first principal component as covariates (**Table 2, Figures 5-**

**6**). The CFA37 association remained genome-wide significant in the mixed model ($p_{raw}$ = 2.3 x $10^{-7}$, $p_{genome}$ = 0.01, GIF = 1.03).

A 10 Mb region of CFA37 from g.5087629 – 15516617 bp (**Figure 7**) was selected for haplotype analysis. The top 5 disease-associated haplotypes reside within a 3 Mb region spanning from g.7061552 – 10653612 bp (**Table 3**) that encompasses 18 protein-coding genes (**Table 4**); the top positional candidate gene in the region is a plasma membrane solute carrier gene, *SLC39A10* (g.8484033 – 8513111 bp).

*Miniature Schnauzer GWAS II (expanded population)*

Miniature Schnauzer GWAS II included 79 cases (≤ 10 yo, 68 confirmed and 11 presumptive) and 47 controls (≥ 9 yo). Association analysis did not identify SNPs with genome-wide significance for any of the three inheritance models (**Table 5**). Results were scattered, and the chromosomal location of the top SNP varied between inheritance models **(Figures 8-10)**. The additive model had an unacceptable $GIF_{chi}$ of 1.16 (**Figure 11**), thus a mixed model was performed with sex and the first principal component as covariates (**Table 6, Figures 12-13**). No SNPs achieved genome-wide significance in the mixed model. Haplotype analysis was not performed due to the absence of a strong association signal.

*Miniature Schnauzer GWAS III (refined population)*

Using stricter phenotyping criteria, a subset of 92 Miniature Schnauzer dogs from GWAS II was selected for GWAS III. The analysis included 49 cases (≤ 8 yo, all

confirmed) and 43 controls (≥ 10 yo). An association signal was present on CFA15, and the top two SNPs in the dominant model had marginal significance at the genome-wide level (BICF2G630433325, g.32567886 bp and BICF2G630433322 g.32570491 bp, $p_{genome} = 0.1$; **Table 7, Figures 14-16**). A mixed model approach was not necessary due to acceptable GIF ≤ 1.10 for all models (**Figure 17**). Haplotype analysis was not performed, but the region containing the top SNPs were evaluated in a subsequent analysis (see the multibreed GWAS).

*Bichon Frise GWAS*

The Bichon Frise GWAS included 16 cases (≤ 8 yo, all confirmed) and 10 controls (≥ 10 yo). Though the population was small, a SNP on CFA15 reached genome-wide significance in the dominant model (BICF2G630431491, g.45744168 bp, $p_{raw} = 9.7 \times 10^{-4}$, $p_{genome} = 0.05$) and a nearby SNP had a significant association in the recessive model (TIGRP2P201486_rs8413090, g.45005683 bp, $p_{raw} = 0.0023$, $p_{genome} = 0.03$; **Table 8, Figures 18-20**). The GIF was ≤ 1.10 for each of the three inheritance models (**Figure 21**), and a mixed model analysis was not performed.

A 10 Mb region of CFA15 from g.40002275 – 50000779 bp (**Figure 22**) was used in the haplotype analysis. The top 5 disease-associated haplotypes span a 5 Mb region from g.42879417 – 47864748 bp (**Table 9**) that encompasses 48 protein-coding genes (**Table 10**); the top positional candidate genes are plasma membrane solute carriers, *SLC5A8* (g.43194640 – 43249817 bp) and *SLC10A7* (47,733,875-47,970,994).

*Multibreed (Miniature Schnauzer and Bichon Frise) GWAS*

The multibreed GWAS included the 65 cases (≤ 8 yo, all confirmed) and 53 controls (≥ 10 yo) from Miniature Schnauzer GWAS III and the Bichon Frise GWAS. A strong signal of association was identified on CFA15, reaching genome-wide significance for multiple SNPs (top SNP = BICF2G630432929, g.33089022 bp, $p_{raw}$ = 8.7 x $10^{-8}$, $p_{genome}$ = 0.005, **Table 11, Figure 24**). The analysis had a high $GIF_{chi}$ at 1.16 and $GIF_{reg}$ at 1.18 (**Figure 23**). Thus, a mixed model was performed with sex and the first principal component as covariates (**Table 12, Figures 25-26**). Three CFA15 SNPs remained genome-wide significant in the mixed model (same top SNP, $p_{raw}$ = 1.6 x $10^{-7}$, $p_{genome}$ = 0.004, GIF = 1.06).

A 20 Mb region of CFA15 from g.28115619 – 48666800 bp (**Figure 27**) was analyzed for haplotype blocks associated with disease. Two regions, approximately 10 Mb apart, contained the top haplotype blocks (**Table 13**). The multibreed region 1 matched the region associated with disease in the Bichon Frise GWAS, and the genes in this region have already been listed (**Table 10**). Region 2 correlated to the disease-associated SNPs identified in Miniature Schnauzer GWAS III. It spans from g.32320032 – 33584438 bp and encompasses 6 genes (**Table 14**); the top positional candidate gene is a calcium-transporting plasma membrane ATPase, *ATP2B1* (g.33559979 – 33629393 bp).

The haplotypes were visually inspected, and a risk haplotype was identified in multibreed region 1 (**Table 10**) that was independently associated with disease in each breed. The risk haplotype had 90% similarity (based on SNP genotypes) between the two

breeds and spanned several of the smaller haplotypes that had been called by Haploview for the Bichon Frise and multibreed analyses. It ran from g.42780328 – 43701090 bp and fit with a dominant inheritance model. Eighty-one percent (13/16) of the Bichon Frise cases had at least one copy of the haplotype compared to only a single female control ($p = 7.8 \times 10^{-4}$ ). Twenty percent (10/49) of the Miniature Schnauzer cases had at least one copy of the haplotype, and the haplotype was not found in any of the Miniature Schnauzer controls ($p = 0.0014$). The association was strengthened by a joint breed analysis with the CMH test ($p = 1.2 \times 10^{-5}$, OR = 80).

Multibreed region 2 (**Table 14**) was also visually inspected for risk haplotypes. Haplotype block 36 of the multibreed analysis (**Table 13)** was the most interesting and fit a recessive inheritance model. This matches with the observation that SNPs in this region had negative ORs in the dominant model, as they tagged a haplotype that was common in the control group. Homozygosity for the risk haplotype across block 36 was significantly associated with stone status in Miniature Schnauzers (25/49 cases were homozygous versus 4/43 controls, $p = 1.4 \times 10^{-5}$), but the haplotype was rare in the Bichons and not significantly associated with disease (2/16 cases were homozygous versus 0/10 controls, $p = 0.5$). Thus the association in the multibreed CMH test ($p = 2.6 \times 10^{-5}$) was slightly less strong than for Miniature Schnauzers alone.

*Miniature Schnauzer GWAS III with CFA15 region 1 as a covariate*

Miniature Schnauzer GWAS III was repeated using the multibreed CFA15 risk haplotype from region 1 (described above) as a covariate, along with sex. Results were

similar to before (**Table 15, Figures 28 – 31**). The same two SNPs from CFA15 region 2 attained marginal significance in the additive model. The other models did not achieve significant signals, but the order of the top SNPs changed. Of note, 4 of the top 5 SNPs in the recessive model resided within the CFA37 region associated with disease in Miniature Schnauzer GWAS I, and the OR for these SNPs were high (OR = 9.5 - 27).

## Discussion

This study reports three loci associated with the trait of CaOx urolithiasis in dogs: one on CFA37 and two on CFA15. CaOx urolithiasis is a complex trait in people, and it is not unexpected that multiple loci are involved in the canine disease as well. However, several factors confound the interpretation of the results at this stage and raise points about replication studies and multibreed GWAS approaches.

The first GWAS included 103 Miniature Schnauzer dogs. It identified a clear disease-associated locus on CFA37 that reached genome-wide significance, even after correction for population stratification. Canine GWAS have been used in the past to identify mutations for incompletely penetrant diseases with fewer than 60 dogs (69, 70), and there was no initial concern that the study population was inadequate or the findings erroneous. Yet, false positive associations are always a risk. There have been many instances in human and veterinary studies where genotype-phenotype associations failed replication in an independent population (120-122). Therefore, an additional small cohort of 23 Miniature Schnauzer dogs was genotyped with the goal of strengthening the preliminary GWAS findings and better defining the top risk haplotype on CFA37.

Unexpectedly, the locus nearly disappeared in the GWAS for this expanded Miniature Schnauzer population of 126 dogs. The third disease-associated SNP in the additive analysis was located on the previously identified region of CFA37, but none of the SNPs even reached marginal significance.

A few explanations were considered for the disappearance of the CFA37 association signal. The first was that the phenotyping criteria may have been insufficiently strict. If there were misclassified dogs in the additional small cohort, their presence may have hidden a true association. CaOx urolithiasis is the Miniature Schnauzer population is presumed to be due to an underlying genetic trait of familial idiopathic hypercalciuria (IH), as described in **Chapter 3**. But there are environmental factors, drug exposures, and disease states that can induce hypercalciuria and cause stones to form in a dog that is free of genetic risk factors. Thus, phenocopies are likely present in the case group. Phenotyping errors are also expected in the control group. CaOx urolithiasis is a late-onset disease in dogs with a mean age at stone diagnosis of 8 yo (57). Using a control age cut-off close to the mean case age runs a high risk that there will be dogs in the control group that could go on to form stones (i.e. latent cases). Furthermore, just as there are environmental factors that can increase risk for stones, there are also those that could lower risk in a genetically susceptible individual. Dual phenotyping with both stone status and urinary calcium measurements would have helped weed out normocalciuric cases that had formed stones from a past environmental exposure and hypercalciuric controls that hadn't yet formed stones, but it was not feasible due to added expense, absence of an established reference range for urinary calcium in

dogs, and the unknown intra-individual variability for spot urinary calcium measurements in dogs (see **Chapter 3**). As an alternative approach to improve phenotyping accuracy, stricter age cut-offs were selected for both cases and controls for Miniature Schnauzer GWAS III. Rather than uncover the CFA37 locus, this analysis identified a separate disease-associated region on CFA15. The CFA15 locus reached marginal genome-wide significance in the dominant model.

The continued absence of disease-association on CFA37 in a cohort with stricter phenotyping criteria forced consideration of another explanation for the loss of this association signal: the original association may have been a false hit. The larger population size in Miniature Schnauzer GWAS II and improved phenotyping in Miniature Schnauzer GWAS III should have increased the power for detecting a true association. Thus, the original CFA37 association may have been caused by a false positive association due to subject outliers or other population structure that was not corrected by the linear mixed model (123). However, a competing theory was also considered. If CaOx urolithiasis is polygenic in the Miniature Schnauzer breed, the locus with the strongest association could mask other loci with smaller or modifying effects. The CFA37 locus may have been obscured by the CFA15 hits. The evidence for this theory is described below.

As discussed earlier, one method of confirming an association is to replicate it in a completely independent population. In dogs, this can be done using another group of the same breed or by selecting a different breed that also suffers from the disease of interest. Susceptibility loci can vary greatly between dog breeds, but there are important

diseases and phenotypes with a shared genetic basis between breeds. In those diseases with a shared basis, the use of a diverse breed or breeds can be critically important to genetic studies. Canine degenerative myelopathy is a classic example where a multibreed approach was necessary to validate the causal mutation (70). In the initial search for the genetic basis of degenerative myelopathy, a GWAS was performed in the Pembroke Welsh Corgi. A signal of association was present on CFA31, but the top SNP was only moderately disease-associated in this breed and did not reach genome-wide significance ($p_{raw}$ = 1 x 10-5, $p_{genome}$ = 0.2). While the Corgi association alone was relatively weak, a regional candidate gene variant proved to be highly associated with disease when a joint case-control association analysis was performed across 5 different breeds ($p = 3 \times 10^{-19}$). This underscores the value of a multibreed approach.

In the later part of the study, we decided to initiate a multibreed approach. A small group of Bichon Frise dogs were enrolled in the study to determine if any of the putative susceptibility loci in Schnauzers demonstrated disease-association in a second breed. The Bichon Frise dogs were analyzed as an individual breed and were combined with the most strictly phenotyped Miniature Schnauzer cohort (GWAS III) for a multibreed analysis. Interestingly, despite a limited number of dogs, the Bichon Frise GWAS identified a disease-associated locus on CFA15, approximately 10 Mb away from the Miniature Schnauzer GWAS II locus. When the breeds were combined for analysis, the Miniature Schnauzer locus drove the top SNP association, but the top haplotype resided in the Bichon Frise locus. Visual inspection revealed a shared risk haplotype that was independently associated with disease in each of the two breeds. It explained risk for

most of the Bichon Frise cases (81%) and a smaller portion of the Miniature Schnauzer cases (20%), and it was strongly associated with disease in a combined breed analysis.

Since the CFA15 multibreed region 1 haplotype described above only explained stone risk for a minority of the Miniature Schnauzers, Miniature Schnauzer GWAS III was repeated with the CFA15 haplotype as a covariate to determine if additional disease-associated loci could be identified. There were three interesting findings in this final analysis. First, accounting for the haplotype over CFA15 region 1 did not reduce the strength of the association of the CFA15 region 2 SNPs. This suggests that there could be two separate CaOx urolithiasis susceptibility loci on CFA15 in Miniature Schnauzers. Second, in the recessive model, the region of CFA37 that had been associated with disease in Miniature Schnauzer GWAS I returned as the top locus. Though the association for the SNPs on CFA37 did not reach genome-wide significance, the maximum OR increased from 13 to 27. This finding supports the theory that CFA37 contains a true susceptibility locus that is being masked by the stronger effect of the CFA15 region 1 locus.

An important future direction will be verification of the association results for the CFA37 locus and both CFA15 loci in larger cohorts. Evidence that the current analyses may be underpowered is provided by evaluating the Q-Q plots and GIF. There were several analyses, primarily the recessive models, where the GIF was < 1.00. This indicates that the p-value distribution was skewed such that fewer low p-values were observed than expected. A low GIF is not typically considered cause for concern. However, it may be a result of underestimation of the relationship between SNPs and

urolithiasis caused by collinearity with sex. This can occur in any regression where predictors are correlated, and PLINK will not report p-values for SNPs that had a > 0.9 correlation with another predictor. More than two thirds of the cases in each GWAS were male dogs, whereas only half of the controls were male. Thus, there were SNPs in each of the recessive models that were dropped from the analysis due to a relationship between homozygosity for the SNP and sex. As more female cases and male control dogs are added to the study cohort, this problem should resolve, and power of detecting a relationship with the recessive model will increase.

In conclusion, this study reports a locus on CFA15 (**Table 10**) that is independently associated with CaOx urolithiasis in the Miniature Schnauzer and Bichon Frise breeds. This region appears to be the major susceptibility locus for the Bichon Frise. In contrast, stone risk in Miniature Schnauzers may be polygenic with additional putative susceptibility loci found on a separate region of CFA15 (**Table 14**) and a region of CFA37 (**Table 4**). Each of these three regions contains one or more plasma membrane transporter genes (*SLC5A8*, *SLC10A7*, *ATP2B1*, and *SLC39A10*), that could play a role in lithogenesis. Sequencing for variant discovery is warranted. Future studies involving other dog breeds at high risk for CaOx urolithiasis are also being considered to confirm risk loci that are shared by multiple breeds and/or uncover novel loci.

**Footnotes**

[a]Puregene blood core kit, Qiagen Sciences, Germantown, MD

[b]CanineHD BeadChip, Illumina, San Diego, CA

| **Table 1. Miniature Schnauzer CaOx GWAS I (initial population)<br>62 Cases (≤ 10 yo, confirmed and presumptive) and 41 Controls (≥ 9 yo)** | | | | | |
|---|---|---|---|---|---|
| ***CFA*** | ***SNP*** | ***Position*** | ***OR*** | ***p-value*** | ***p-genome*** |
| **Additive model with sex as a covariate, $GIF_{reg}$ = 1.03, $GIF_{chi}$ = 1.13** | | | | | |
| **37** | **BICF2P650021** | **10603162** | **0.12** | **3.6E-06** | **0.001** |
| **37** | **BICF2S23230162** | **10636867** | **0.15** | **9.7E-06** | **0.01** |
| **37** | **BICF2P772008** | **10644633** | **0.15** | **9.7E-06** | **0.01** |
| **37** | **TIGRP2P417891_rs8759697** | **10653612** | **0.15** | **9.7E-06** | **0.01** |
| **37** | **BICF2P983344** | **10096575** | **0.17** | **1.1E-05** | **0.02** |
| 37 | BICF2G630125983 | 9079742 | 0.20 | 4.5E-05 | 0.12 |
| 37 | BICF2S23759836 | 9043858 | 0.21 | 5.8E-05 | 0.18 |
| 37 | BICF2P1300501 | 8632668 | 0.24 | 6.4E-05 | 0.20 |
| 37 | BICF2G630125940 | 9031081 | 0.23 | 7.0E-05 | 0.22 |
| 37 | BICF2G630127555 | 12406988 | 0.20 | 7.6E-05 | 0.25 |
| **Dominant model with sex as a covariate, $GIF_{reg}$ = 1.03, $GIF_{chi}$ = 1.02** | | | | | |
| 37 | BICF2G630125940 | 9031081 | 0.10 | 3.2E-05 | 0.20 |
| 37 | BICF2S23759836 | 9043858 | 0.11 | 5.9E-05 | 0.37 |
| 18 | BICF2P1325569 | 25476093 | 0.14 | 6.8E-05 | 0.43 |
| 37 | BICF2G630126631 | 10489812 | 0.12 | 8.9E-05 | 0.55 |
| 37 | BICF2P983344 | 10096575 | 0.080 | 9.3E-05 | 0.57 |
| 37 | BICF2G630125583 | 8535368 | 0.13 | 9.9E-05 | 0.60 |
| 37 | BICF2G630126132 | 9237150 | 0.13 | 0.00012 | 0.69 |
| 37 | BICF2S2292150 | 9207753 | 0.14 | 0.00014 | 0.74 |
| 37 | BICF2S2292221 | 9211968 | 0.14 | 0.00014 | 0.74 |
| 37 | BICF2G630125983 | 9079742 | 0.12 | 0.00014 | 0.75 |
| **Recessive model with sex as a covariate, $GIF_{reg}$ = <1.00, $GIF_{chi}$ < 1.00** | | | | | |
| **37** | **BICF2P650021** | **10603162** | **0.062** | **1.9E-05** | **0.007** |
| **37** | **BICF2S23230162** | **10636867** | **0.092** | **9.7E-05** | **0.06** |
| **37** | **BICF2P772008** | **10644633** | **0.092** | **9.7E-05** | **0.06** |
| **37** | **TIGRP2P417891_rs8759697** | **10653612** | **0.092** | **9.7E-05** | **0.06** |
| 37 | BICF2G630126183 | 9265505 | 13 | 0.00037 | 0.42 |
| 37 | BICF2G630126206 | 9277394 | 13 | 0.00037 | 0.42 |
| 37 | BICF2P1441475 | 9302247 | 13 | 0.00037 | 0.42 |
| 37 | BICF2G630126227 | 9307151 | 13 | 0.00037 | 0.42 |
| 37 | BICF2G630126238 | 9322804 | 13 | 0.00037 | 0.42 |
| 37 | BICF2P983344 | 10096575 | 0.13 | 0.00050 | 0.56 |

SNPs that were met genome-wide significance ($p < 0.05$) are bolded in blue font; those with marginal significance ($p < 0.1$) are in bolded in black font.

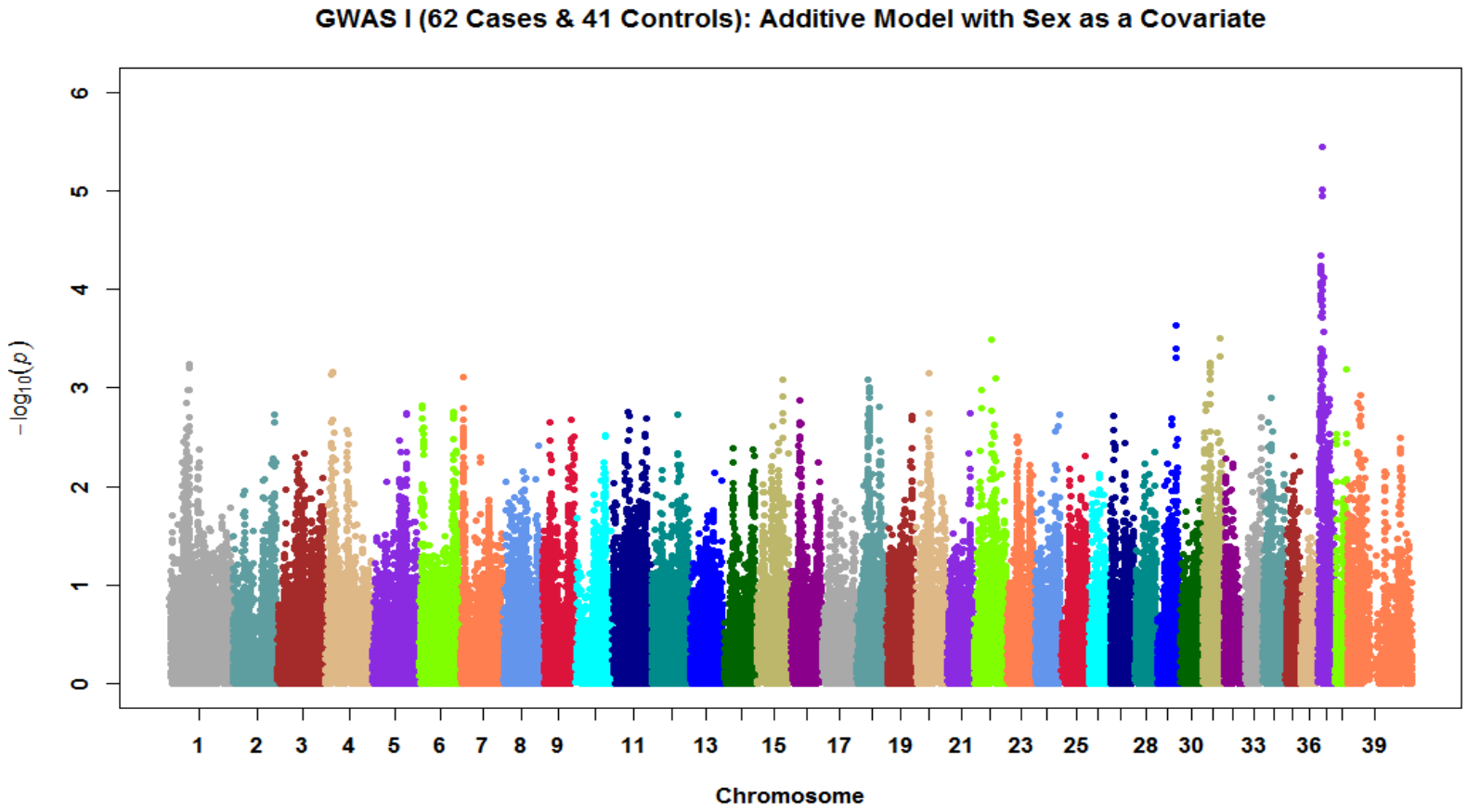



**Figure 1.** Manhattan plot for Miniature Schnauzer GWAS I including 62 dogs with a history of CaOx urolithiasis (cases) and 41 stone-free dogs (controls). The plot displays results of an additive model with sex as a covariate.

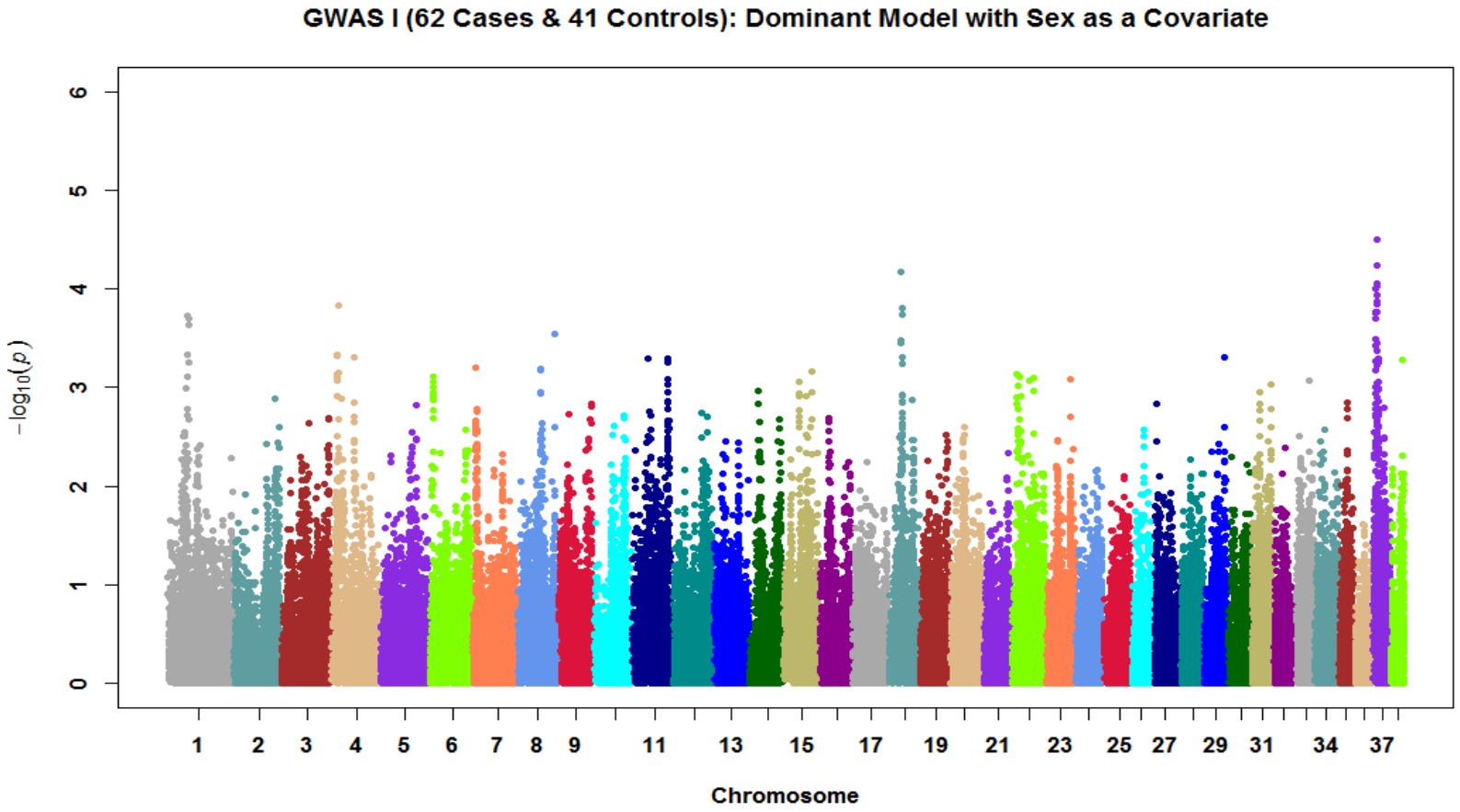



**Figure 2.** Manhattan plot for Miniature Schnauzer GWAS I including 62 dogs with a history of CaOx urolithiasis (cases) and 41 stone-free dogs (controls). The plot displays results of a dominant model with sex as a covariate.

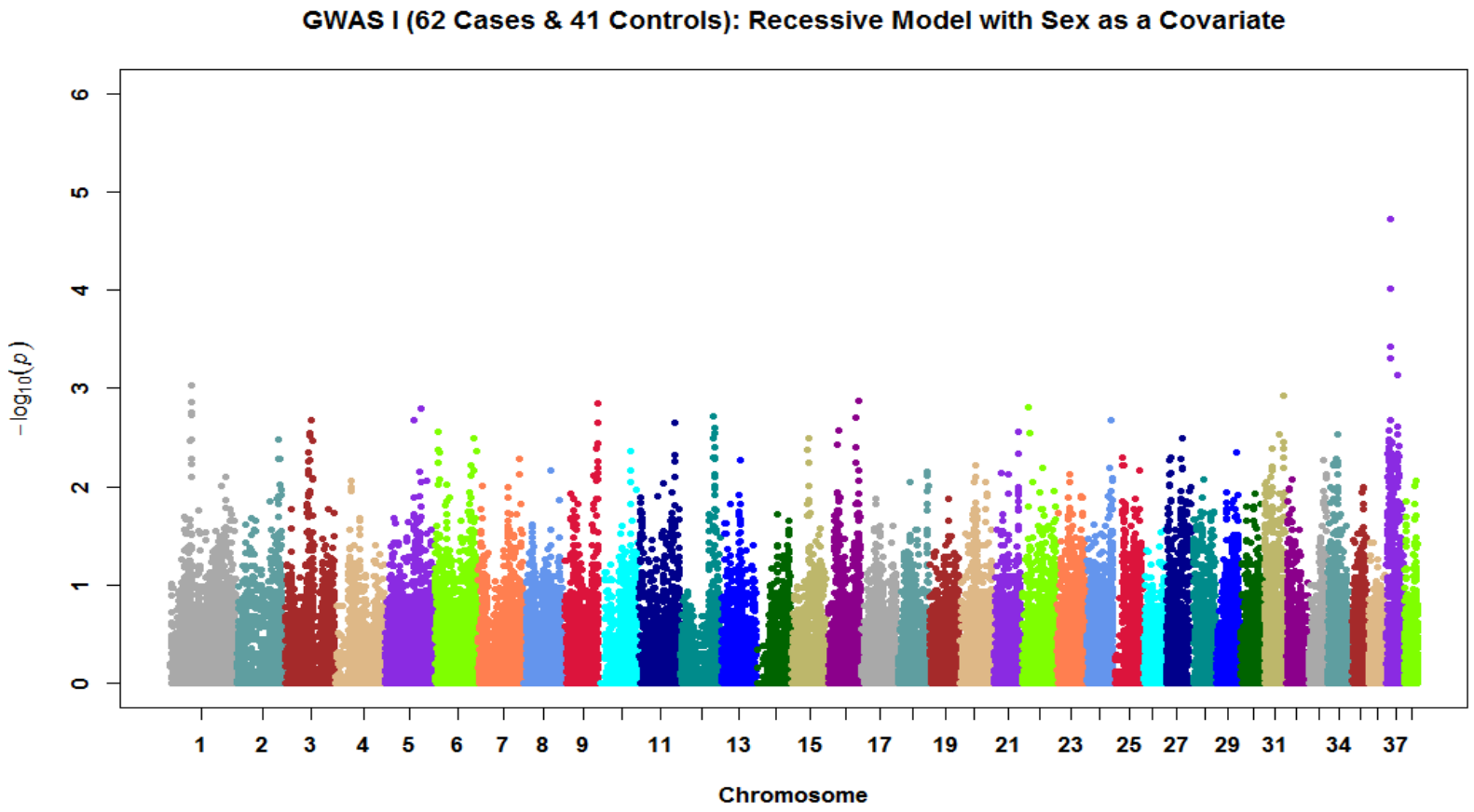



**Figure 3.** Manhattan plot for Miniature Schnauzer GWAS I including 62 dogs with a history of CaOx urolithiasis (cases) and 41 stone-free dogs (controls). The plot displays results of a recessive model with sex as a covariate.

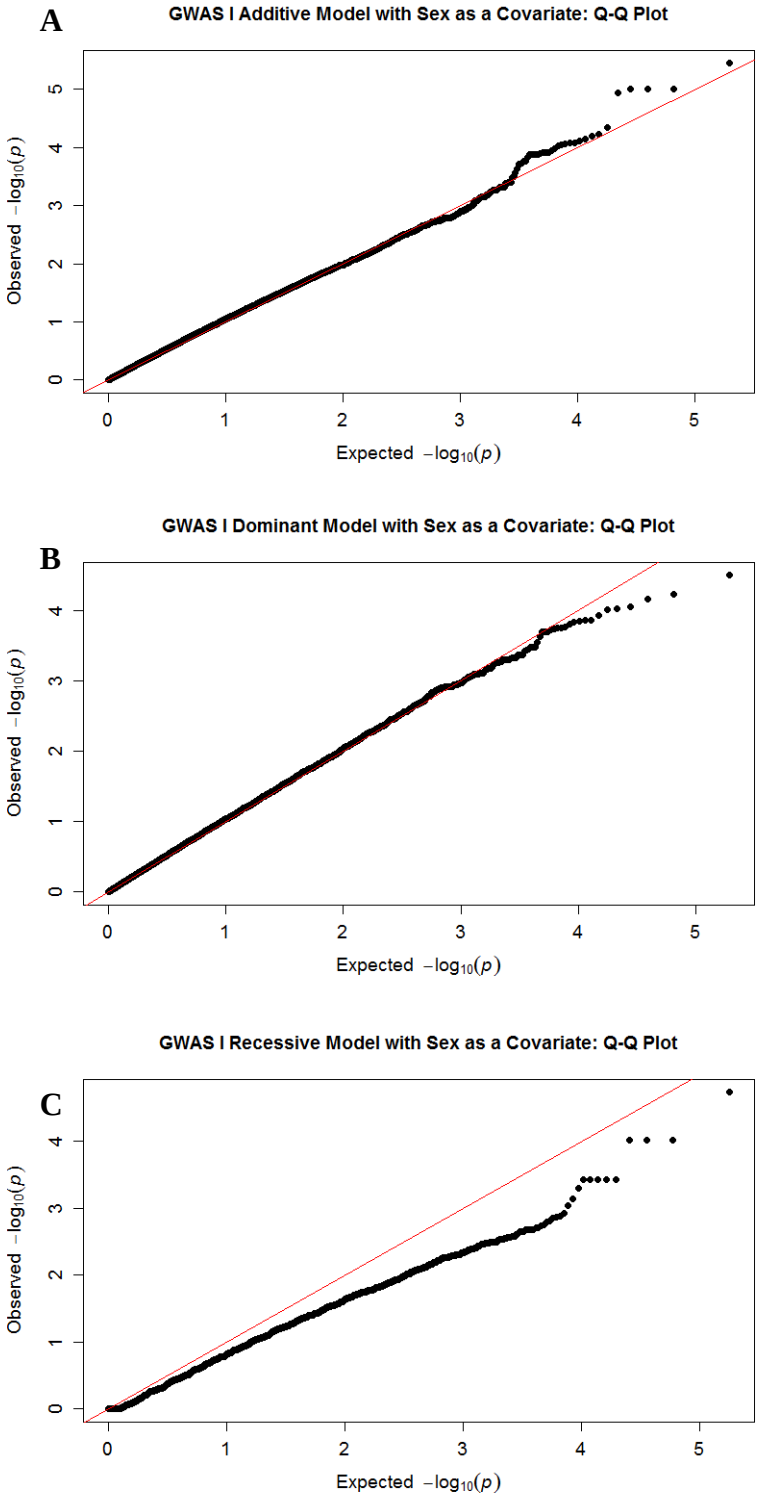



**Figure 4.** Q-Q plots for Miniature Schnauzer GWAS I: A) additive model $GIF_{reg}$ = 1.03, B) dominant model $GIF_{reg}$ = 1.03, and C) recessive model $GIF_{reg}$ < 1.00.

| Table 2. Miniature Schnauzer CaOx GWAS I (initial population) GenABEL mixed model with the 1st PCA and sex as covariates, GIF = 1.03 | | | | | |
|---|---|---|---|---|---|
| ***CFA*** | ***SNP*** | ***Position*** | ***B allele effect*** | ***p-value*** | ***p-genome*** |
| **37** | **BICF2P650021** | **10603162** | **-0.33** | **2.3E-07** | **0.010** |
| **37** | **BICF2S23230162** | **10636867** | **-0.31** | **1.2E-06** | **0.069** |
| **37** | **BICF2P772008** | **10644633** | **-0.31** | **1.2E-06** | **0.069** |
| **37** | **TIGRP2P417891_rs8759697** | **10653612** | **-0.31** | **1.2E-06** | **0.069** |
| **37** | **BICF2P983344** | **10096575** | **-0.29** | **1.5E-06** | **0.069** |
| 37 | BICF2S23759836 | 9043858 | -0.29 | 7.9E-06 | 0.31 |
| 37 | BICF2G630125983 | 9079742 | -0.29 | 8.8E-06 | 0.46 |
| 37 | BICF2G630125940 | 9031081 | -0.28 | 7.0E-05 | 0.49 |
| 37 | BICF2P1300501 | 8632668 | -0.27 | 6.4E-05 | 0.64 |
| 37 | BICF2P993759 | 11812177 | -0.29 | 1.8E-05 | 0.75 |

SNPs that were met genome-wide significance ($p < 0.05$) are bolded in blue font; those with marginal significance ($p < 0.1$) are in bolded in black font.

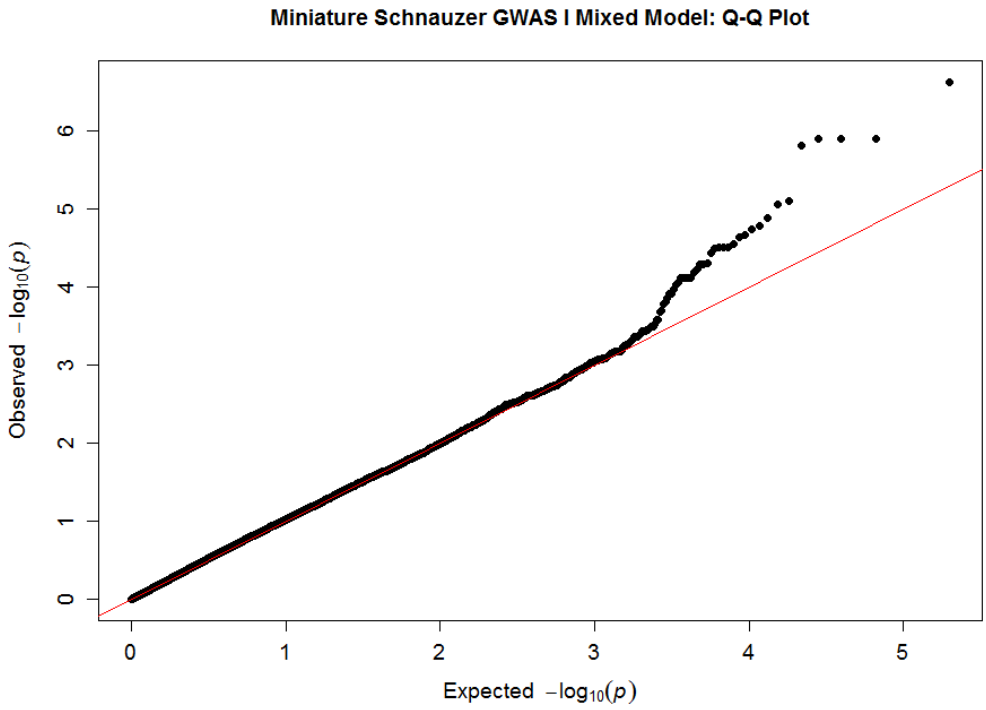



**Figure 5.** Q-Q plot for Miniature Schnauzer GWAS I mixed model analysis with the 1st principal component and sex as covariates, GIF = 1.03.

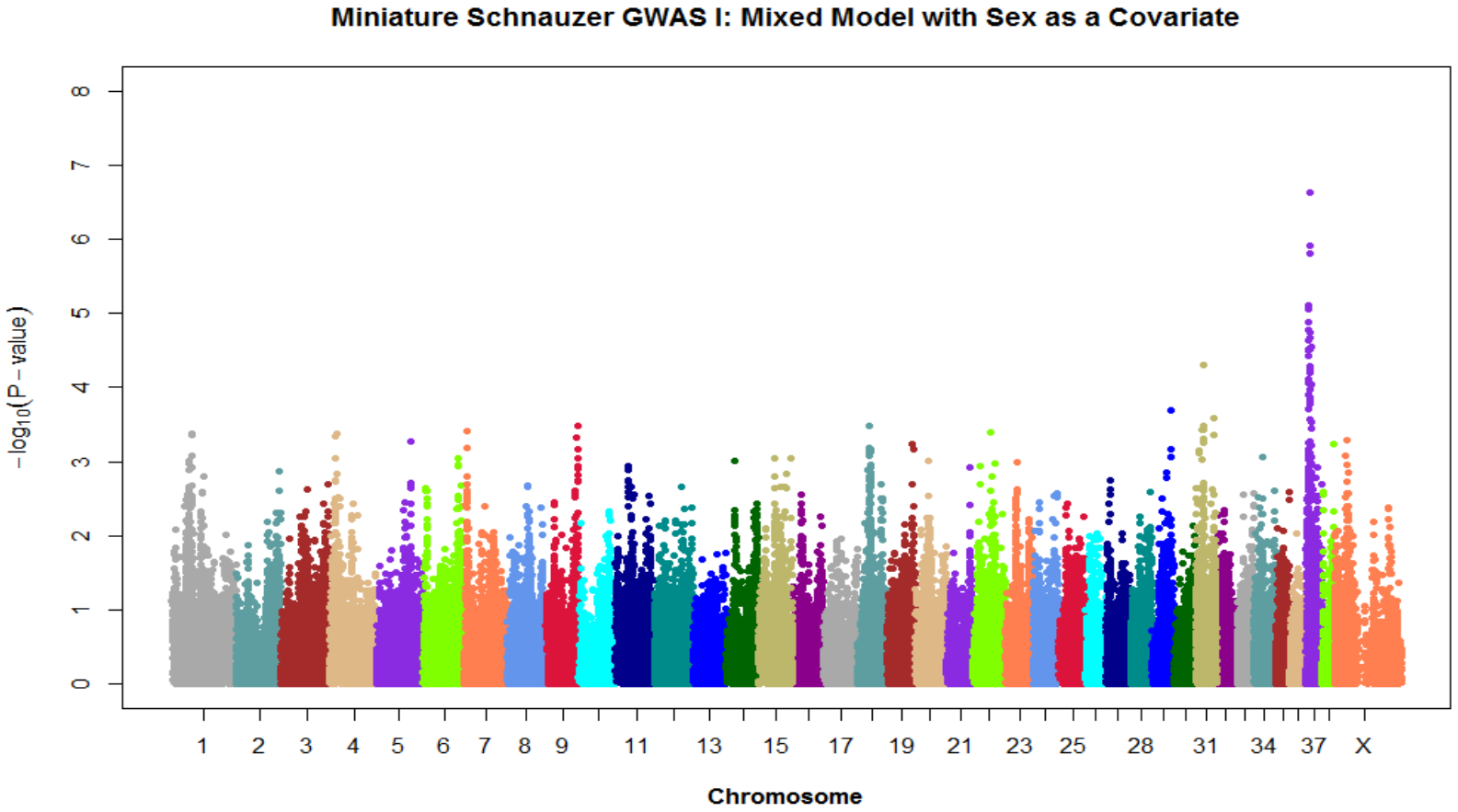



**Figure 6.** Manhattan plot for Miniature Schnauzer GWAS I including 62 dogs with a history of CaOx urolithiasis (cases) and 41 stone-free dogs (controls). The plot displays results of a mixed model with the 1st principal component and sex as covariates.

| **Table 3. Miniature Schnauzer CaOx GWAS I (initial population)<br>CFA37 top 5 haplotype blocks (out of 68 total spanning a 10 Mb region)** | | | | | |
|---|---|---|---|---|---|
| ***Block*** | ***Start*** | ***End*** | ***Case, Control Frequency*** | ***OR*** | ***p-value*** |
| 34 | 10489812 | 10583353 | 0.46, 0.17 | 4.1 | 1.9E-05 |
| 35 | 10615627 | 10653612 | 0.46, 0.18 | 3.8 | 4.5E-05 |
| 27 | 9006590 | 9043858 | 0.44, 0.17 | 3.9 | 4.9E-05 |
| 16 | 7378229 | 7876584 | 0.44, 0.17 | 3.9 | 4.9E-05 |
| 13 | 7061552 | 7177364 | 0.43, 0.17 | 3.7 | 7.6E-05 |

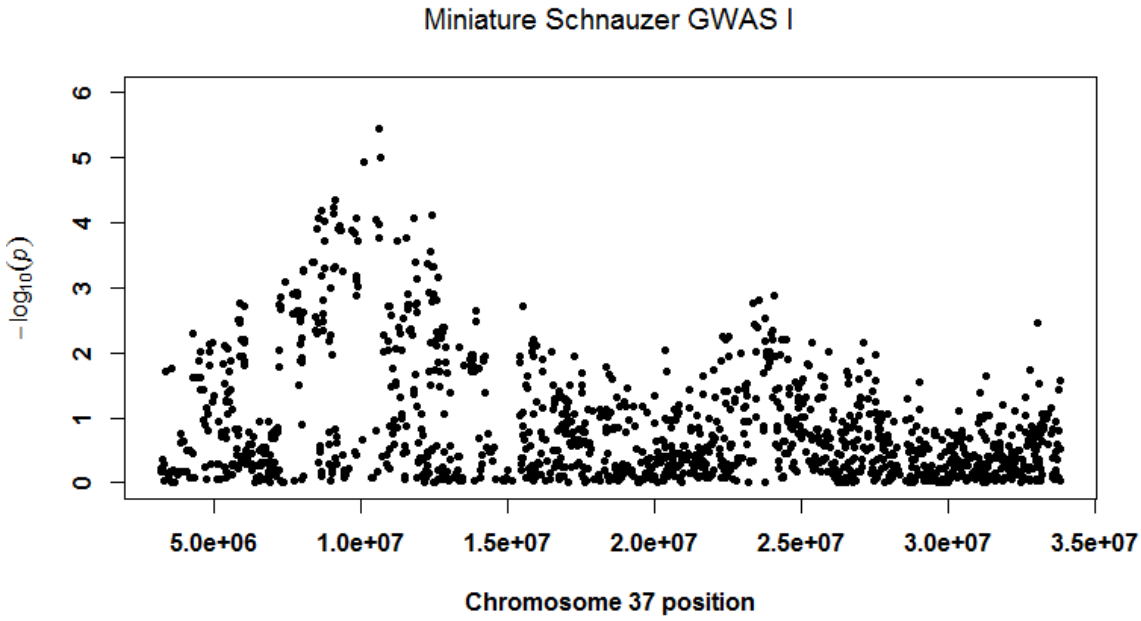



**Figure 7.** Results for a 10 Mb region on CFA37 that encompasses the top SNPs associated with CaOx urolithiasis in Miniature Schnauzer GWAS I.

| **Table 4. Miniature Schnauzer CaOx GWAS I<br>Protein-coding genes in the top haplotype region of CFA37<br>g.7061552 – 10653612 bp** | | | | | |
|---|---|---|---|---|---|
| ***SLC39A10*** | *DNAH7* | *STK17B* | *HECW2* | *CCDC150* | *GTF3C3* |
| *C2orf66* | *PGAP1* | *ANKRD44* | *SF3B1* | *COQ10B* | *HSPD1* |
| *HSPE1* | *MOB4* | *RFTN2* | *MARS2* | *BOLL* | *PLCL1* |

Top candidate gene based on function and tissue localization is bolded in blue font.

| **Table 5. Miniature Schnauzer CaOx GWAS II (expanded population) 79 Cases (≤ 10 yo, confirmed and presumptive) and 47 Controls (≥ 9 yo)** | | | | | |
|---|---|---|---|---|---|
| ***CFA*** | ***SNP*** | ***Position*** | ***OR*** | ***p-value*** | ***p-genome*** |
| **Additive model with sex as a covariate, $GIF_{reg}$ = 1.07, $GIF_{chi}$ = 1.16** | | | | | |
| 39 | BICF2P131020 | 24776612 | 145 | 0.00011 | 0.50 |
| 1 | BICF2S2298939 | 33203114 | 0.21 | 0.00012 | 0.58 |
| 37 | TIGRP2P423581_rs8897643 | 8736596 | 0.31 | 0.00015 | 0.65 |
| 39 | BICF2P673846 | 24296885 | 0.23 | 0.00025 | 0.84 |
| 18 | TIGRP2P246440_rs8532180 | 28334275 | 0.22 | 0.00025 | 0.85 |
| 1 | BICF2G630723610 | 42908847 | 3.6 | 0.00027 | 0.87 |
| 39 | BICF2G630534063 | 24598551 | 7.6 | 0.00028 | 0.88 |
| 39 | BICF2G630534027 | 24662216 | 6.4 | 0.00029 | 0.88 |
| 1 | BICF2G630723607 | 42903982 | 3.5 | 0.00030 | 0.89 |
| 18 | BICF2P946872 | 28424428 | 0.23 | 0.00030 | 0.89 |
| **Dominant model with sex as a covariate, $GIF_{reg}$ = 1.06, $GIF_{chi}$ = 1.05** | | | | | |
| 18 | BICF2P1325569 | 25476093 | 0.15 | 1.6E-05 | 0.16 |
| 18 | BICF2P263188 | 26565593 | 0.17 | 5.2E-05 | 0.46 |
| 18 | TIGRP2P245824_rs8664446 | 25468973 | 0.18 | 5.3E-05 | 0.47 |
| 18 | BICF2P1359352 | 25488478 | 0.18 | 5.3E-05 | 0.47 |
| 18 | BICF2S23440466 | 26591536 | 0.18 | 6.5E-05 | 0.55 |
| 1 | BICF2S2298939 | 33203114 | 0.16 | 6.9E-05 | 0.57 |
| 18 | BICF2P488325 | 25944658 | 0.19 | 9.1E-05 | 0.67 |
| 1 | BICF2G630723610 | 42908847 | 5.3 | 9.4E-05 | 0.68 |
| 1 | BICF2G630723607 | 42903982 | 5.2 | 0.00011 | 0.75 |
| 18 | TIGRP2P245473_rs8568436 | 26643716 | 0.20 | 0.00011 | 0.77 |
| **Recessive model with sex as a covariate, $GIF_{reg}$ = < 1.00, $GIF_{chi}$ < 1.00** | | | | | |
| 3 | BICF2S23419889 | 48557713 | 0.12 | 0.00015 | 0.27 |
| 37 | BICF2P457642 | 23741393 | 0.16 | 0.00031 | 0.53 |
| 5 | BICF2S23660272 | 55026850 | 0.18 | 0.00032 | 0.54 |
| 3 | TIGRP2P43282_rs8951545 | 48582174 | 0.15 | 0.00040 | 0.66 |
| 3 | BICF2P321592 | 47692430 | 0.16 | 0.00047 | 0.72 |
| 12 | BICF2G630119799 | 62928191 | 0.19 | 0.00053 | 0.76 |
| 12 | BICF2G630119146 | 64046928 | 0.19 | 0.00085 | 0.91 |
| 12 | TIGRP2P170259_rs9174095 | 63441894 | 0.21 | 0.00088 | 0.93 |
| 24 | TIGRP2P321418_rs9212628 | 48117972 | 0.10 | 0.00092 | 0.93 |
| 37 | BICF2S23112989 | 23755956 | 0.19 | 0.00099 | 0.95 |

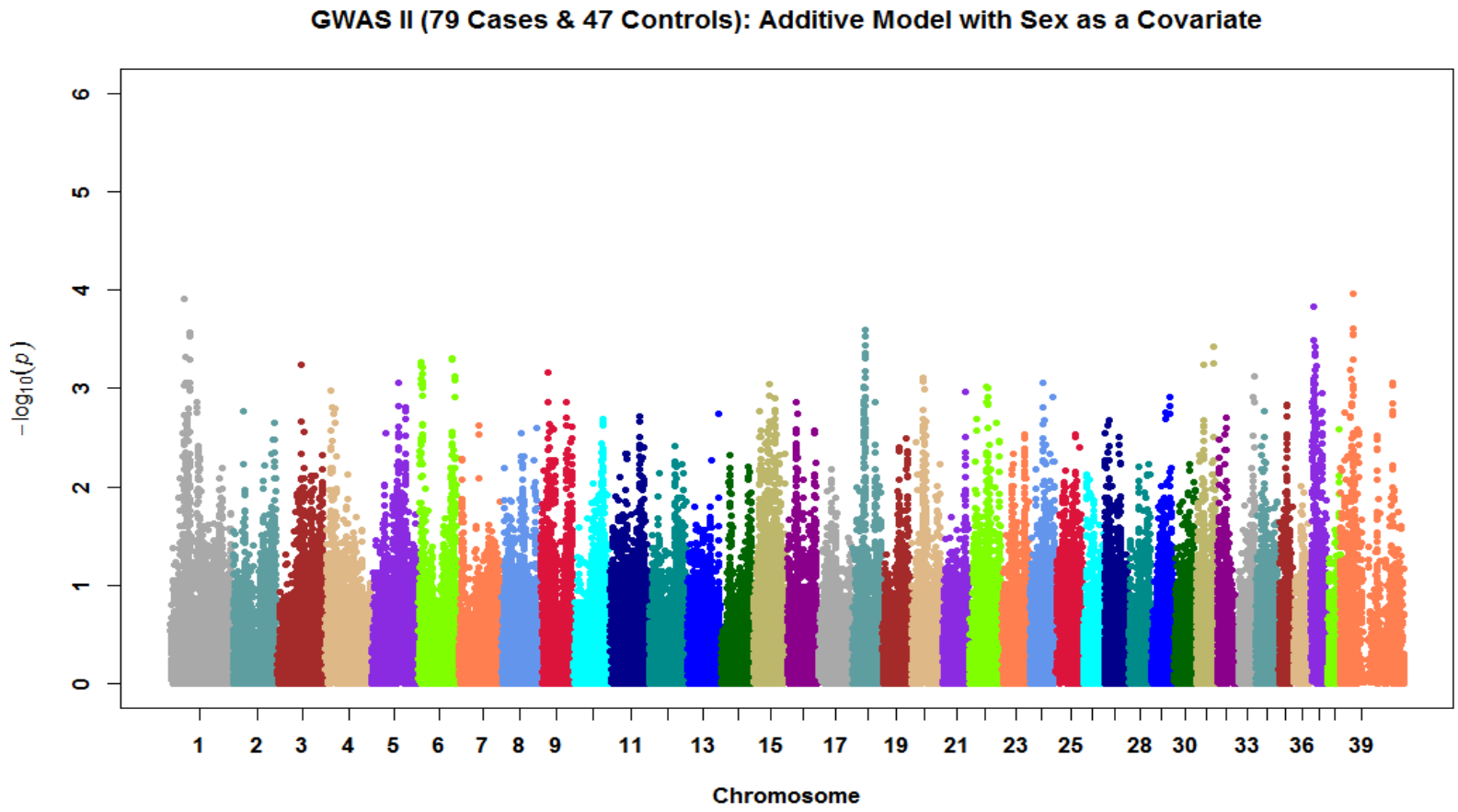



**Figure 8.** Manhattan plot for Miniature Schnauzer GWAS II including 79 dogs with a history of CaOx urolithiasis (cases) and 47 stone-free dogs (controls). The plot displays results of an additive model with sex as a covariate.

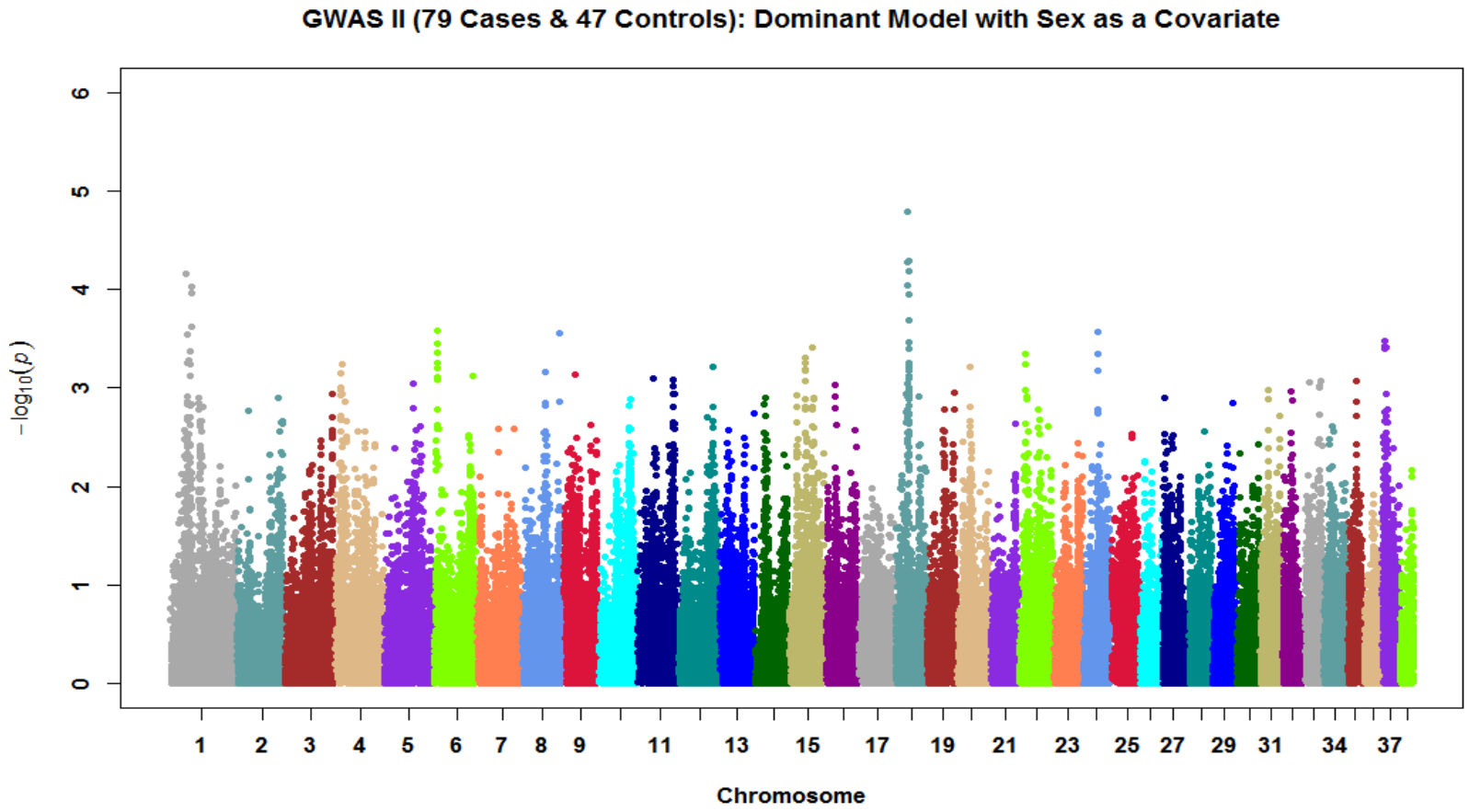



**Figure 9.** Manhattan plot for Miniature Schnauzer GWAS II including 79 dogs with a history of CaOx urolithiasis (cases) and 47 stone-free dogs (controls). The plot displays results of a dominant model with sex as a covariate.

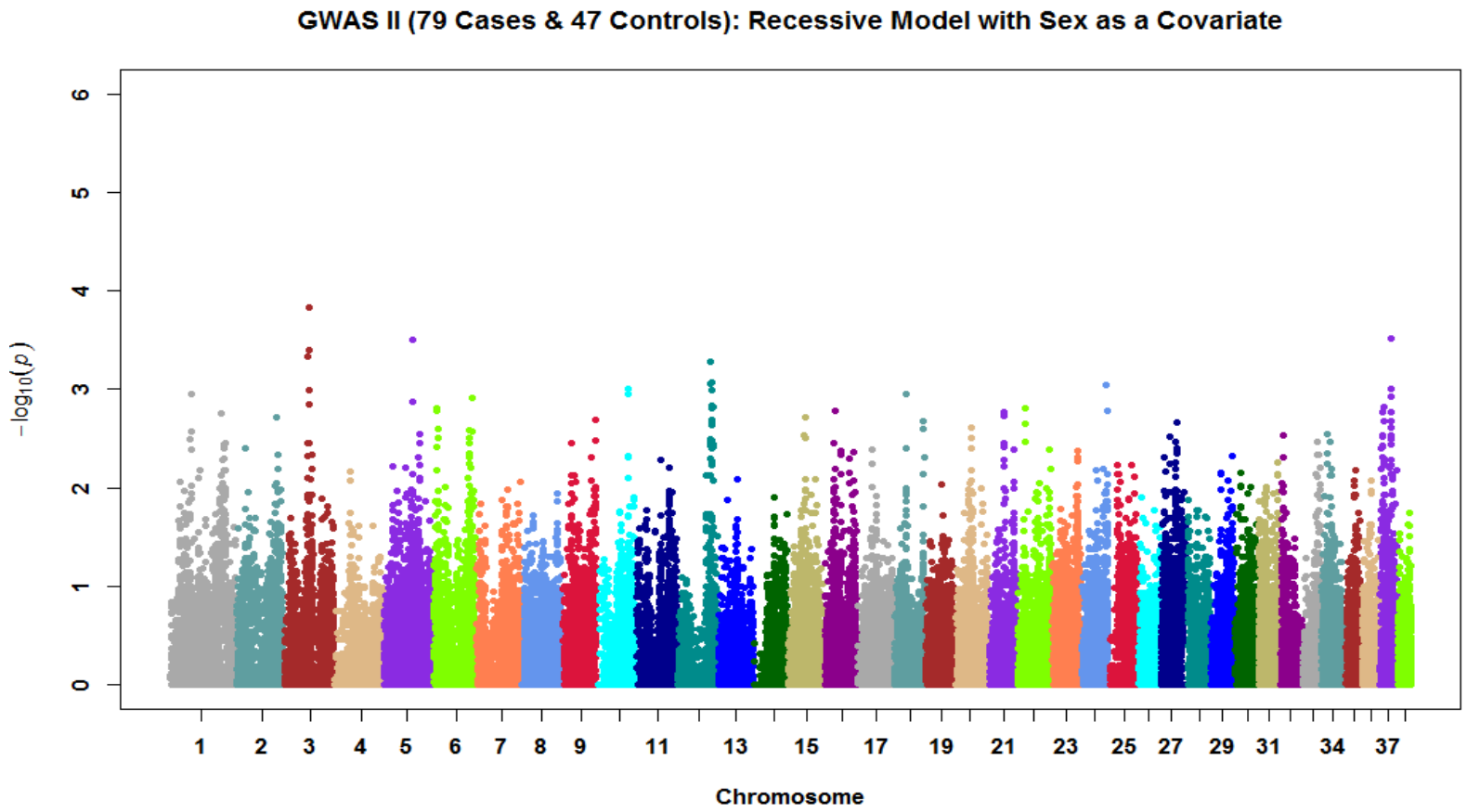



**Figure 10.** Manhattan plot for Miniature Schnauzer GWAS II including 79 dogs with a history of CaOx urolithiasis (cases) and 47 stone-free dogs (controls). The plot displays results of a recessive model with sex as a covariate.

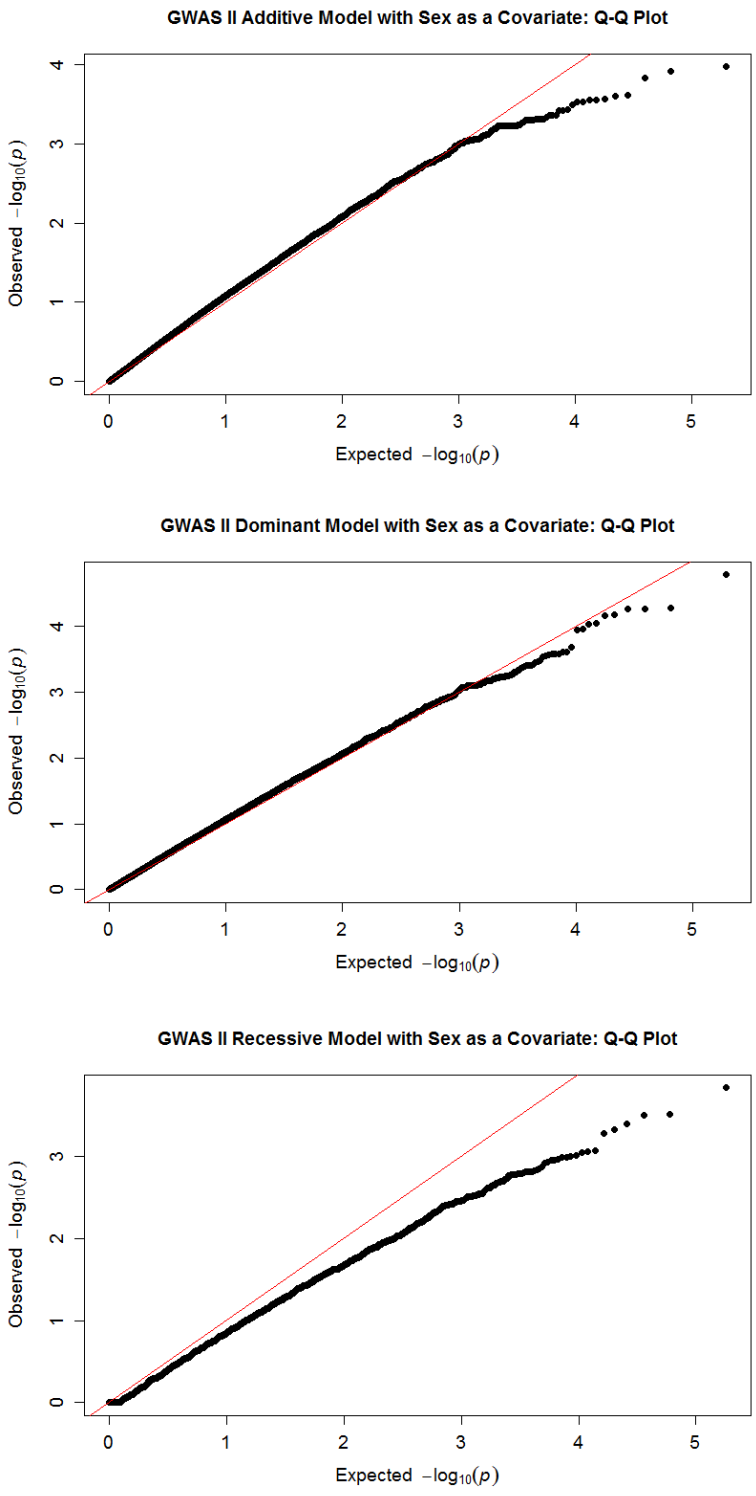



**Figure 11.** Q-Q plots for Miniature Schnauzer GWAS II: A) additive model $GIF_{reg}$ = 1.07, B) dominant model $GIF_{reg}$ = 1.06, and C) recessive model $GIF_{reg}$ < 1.00.

| Table 6. Miniature Schnauzer CaOx GWAS II (expanded population) GenABEL mixed model with the 1st PCA and sex as covariates, GIF = 1.02 | | | | | |
|---|---|---|---|---|---|
| ***CFA*** | ***SNP*** | ***Position*** | ***B allele effect*** | ***p-value*** | ***p-genome*** |
| 1 | BICF2S2298939 | 33203114 | -0.31 | 5.4E-05 | 0.73 |
| 39 | BICF2P673846 | 24296885 | -0.20 | 0.00011 | 0.83 |
| 39 | BICF2P131020 | 24776612 | 0.20 | 0.00015 | 0.99 |
| 18 | BICF2P946872 | 28424428 | -0.32 | 0.00018 | 1.0 |
| 18 | TIGRP2P246440_rs8532180 | 28334275 | -0.32 | 0.00019 | 1.0 |
| 6 | BICF2P1198194 | 68330591 | -0.21 | 0.00020 | 0.81 |
| 1 | BICF2S23543670 | 58104298 | 0.36 | 0.00021 | 0.81 |
| 1 | BICF2P397814 | 58220839 | 0.36 | 0.00021 | 0.99 |
| 6 | BICF2P1198195 | 68330390 | -0.21 | 0.00022 | 0.85 |
| 31 | BICF2P570750 | 38184486 | -0.25 | 0.00023 | 1.0 |

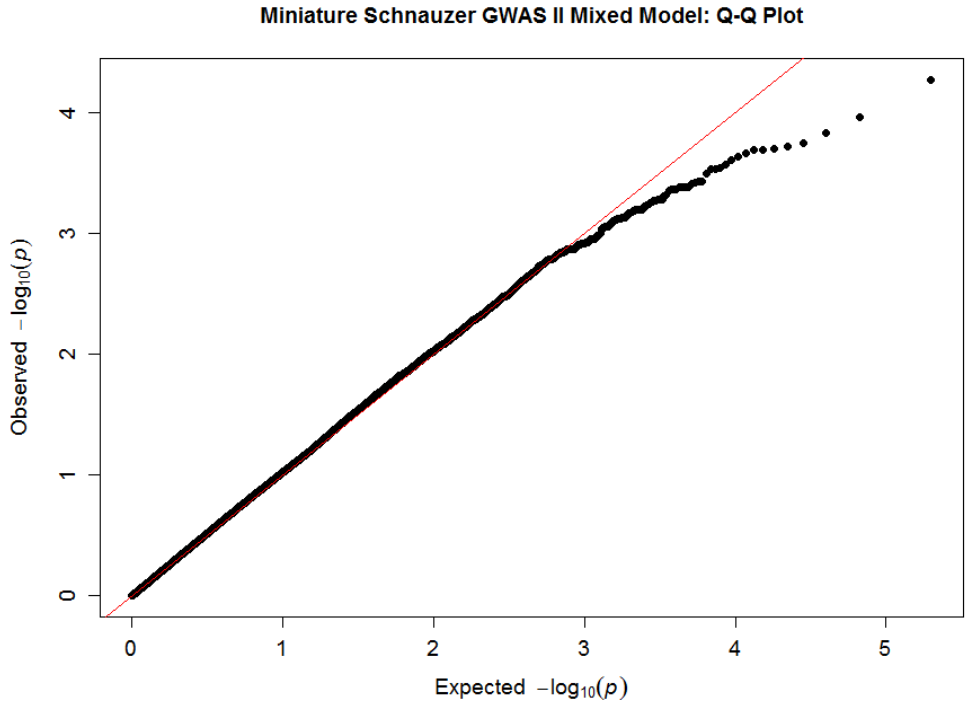



**Figure 12.** Q-Q plot for Miniature Schnauzer GWAS II mixed model analysis with the 1st principal component and sex as covariates, GIF = 1.02.

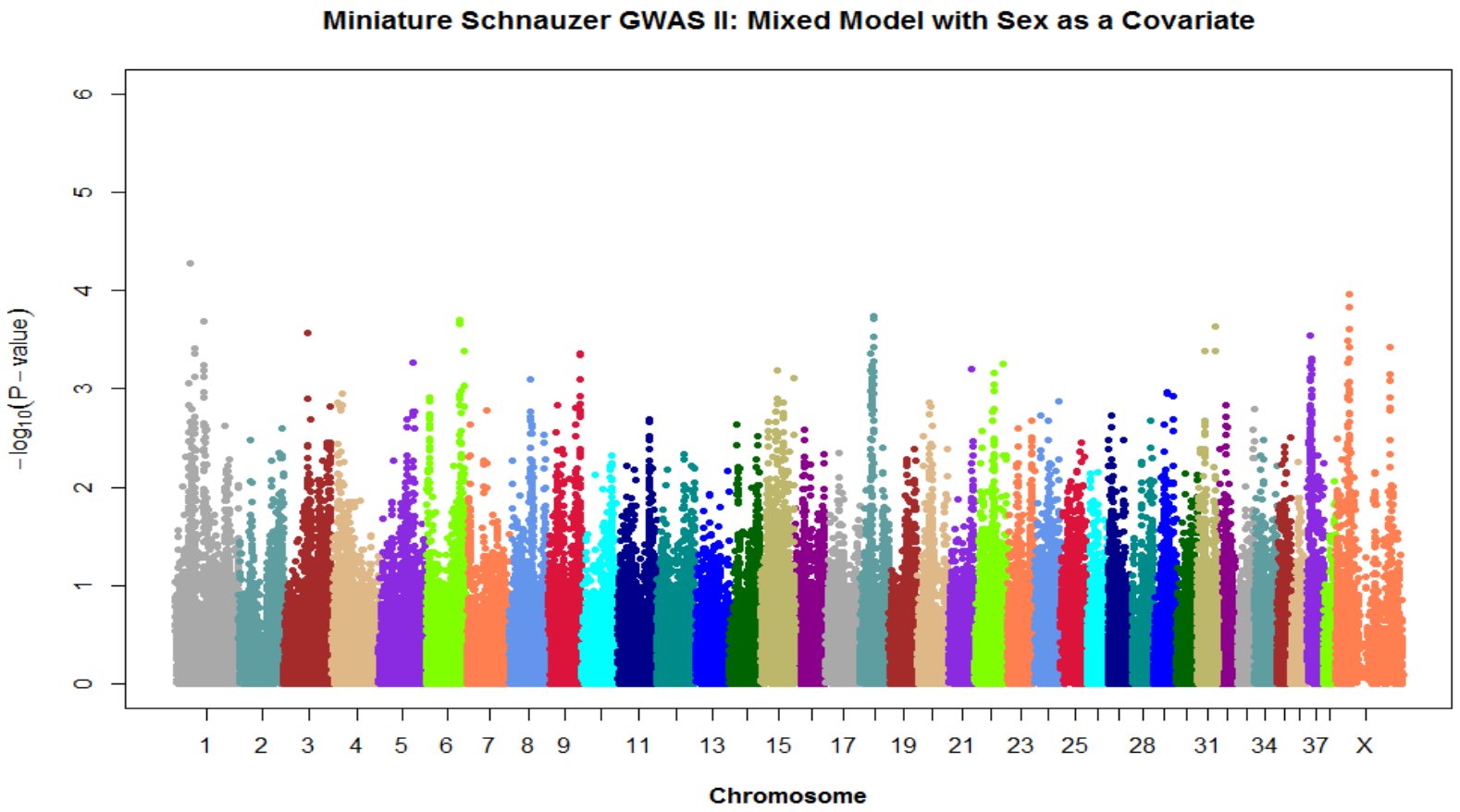



**Figure 13.** Manhattan plot for Miniature Schnauzer GWAS II including 79 dogs with a history of CaOx urolithiasis (cases) and 47 stone-free dogs (controls). The plot displays results of a mixed model with the 1$^{st}$ principal component and sex as covariates.

| **Table 7. Miniature Schnauzer CaOx GWAS III**<br>**49 Cases (≤ 8 yo, confirmed only) and 43 Controls (≥ 10 yo)** | | | | | |
|---|---|---|---|---|---|
| ***CFA*** | ***SNP*** | ***Position*** | ***OR*** | ***p-value*** | ***p-genome*** |
| **Additive model with sex as a covariate, $GIF_{reg}$ = 1.03, $GIF_{chi}$ = 1.10** | | | | | |
| 15 | BICF2G630433325 | 32567886 | 0.23 | 7.2E-05 | 0.21 |
| 15 | BICF2G630433322 | 32570491 | 0.23 | 7.2E-05 | 0.21 |
| 15 | BICF2G630433332 | 32546804 | 0.22 | 8.7E-05 | 0.26 |
| 15 | BICF2G630433396 | 32498694 | 0.24 | 9.4E-05 | 0.29 |
| 15 | BICF2G630433386 | 32503260 | 0.24 | 9.4E-05 | 0.29 |
| 15 | BICF2G630433404 | 32482976 | 0.24 | 9.6E-05 | 0.30 |
| 39 | BICF2P131020 | 24776612 | 21 | 0.00012 | 0.33 |
| 15 | BICF2G630433365 | 32523939 | 0.24 | 0.00012 | 0.37 |
| 15 | BICF2G630433373 | 32513694 | 0.23 | 0.00014 | 0.47 |
| 15 | BICF2P270746 | 32468068 | 0.23 | 0.00017 | 0.57 |
| **Dominant model with sex as a covariate, $GIF_{reg}$ = 1.02, $GIF_{chi}$ = 1.00** | | | | | |
| **15** | **BICF2G630433325** | **32567886** | **0.075** | **2.4E-05** | **0.10** |
| **15** | **BICF2G630433322** | **32570491** | **0.075** | **2.4E-05** | **0.10** |
| 15 | BICF2G630433396 | 32498694 | 0.080 | 3.9E-05 | 0.23 |
| 15 | BICF2G630433386 | 32503260 | 0.080 | 3.9E-05 | 0.23 |
| 15 | BICF2G630433404 | 32482976 | 0.080 | 3.9E-05 | 0.23 |
| 15 | BICF2G630433373 | 32513694 | 0.080 | 4.9E-05 | 0.28 |
| 15 | BICF2G630433365 | 32523939 | 0.085 | 5.2E-05 | 0.29 |
| 15 | BICF2G630433332 | 32546804 | 0.065 | 6.6E-05 | 0.39 |
| 22 | BICF2G630318063 | 14088828 | 0.13 | 8.6E-05 | 0.50 |
| 22 | BICF2G630318057 | 14086646 | 0.14 | 0.00012 | 0.62 |
| **Recessive model with sex as a covariate, $GIF_{reg}$ < 1.00, $GIF_{chi}$ < 1.00** | | | | | |
| 37 | BICF2P457642 | 23741393 | 0.11 | 0.00067 | 0.49 |
| 15 | BICF2G630432586 | 33571267 | 9.8 | 0.00078 | 0.56 |
| 1 | BICF2P717515 | 100616715 | 17 | 0.00091 | 0.65 |
| 6 | G378f45S224 | 10006580 | 13 | 0.00096 | 0.68 |
| 37 | TIGRP2P420378_rs8991216 | 24538218 | 0.14 | 0.0010 | 0.72 |
| 37 | TIGRP2P423581_rs8897643 | 8736596 | 13 | 0.0011 | 0.80 |
| 12 | BICF2P375443 | 65735080 | 0.15 | 0.0011 | 0.81 |
| 6 | BICF2S23740063 | 9560403 | 13 | 0.0013 | 0.87 |
| 6 | BICF2S2373477 | 9978392 | 13 | 0.0013 | 0.87 |
| 23 | BICF2P434381 | 54606608 | 0.15 | 0.0013 | 0.87 |

SNPs with marginal significance ($p < 0.1$) at the genome-wide level are bolded.

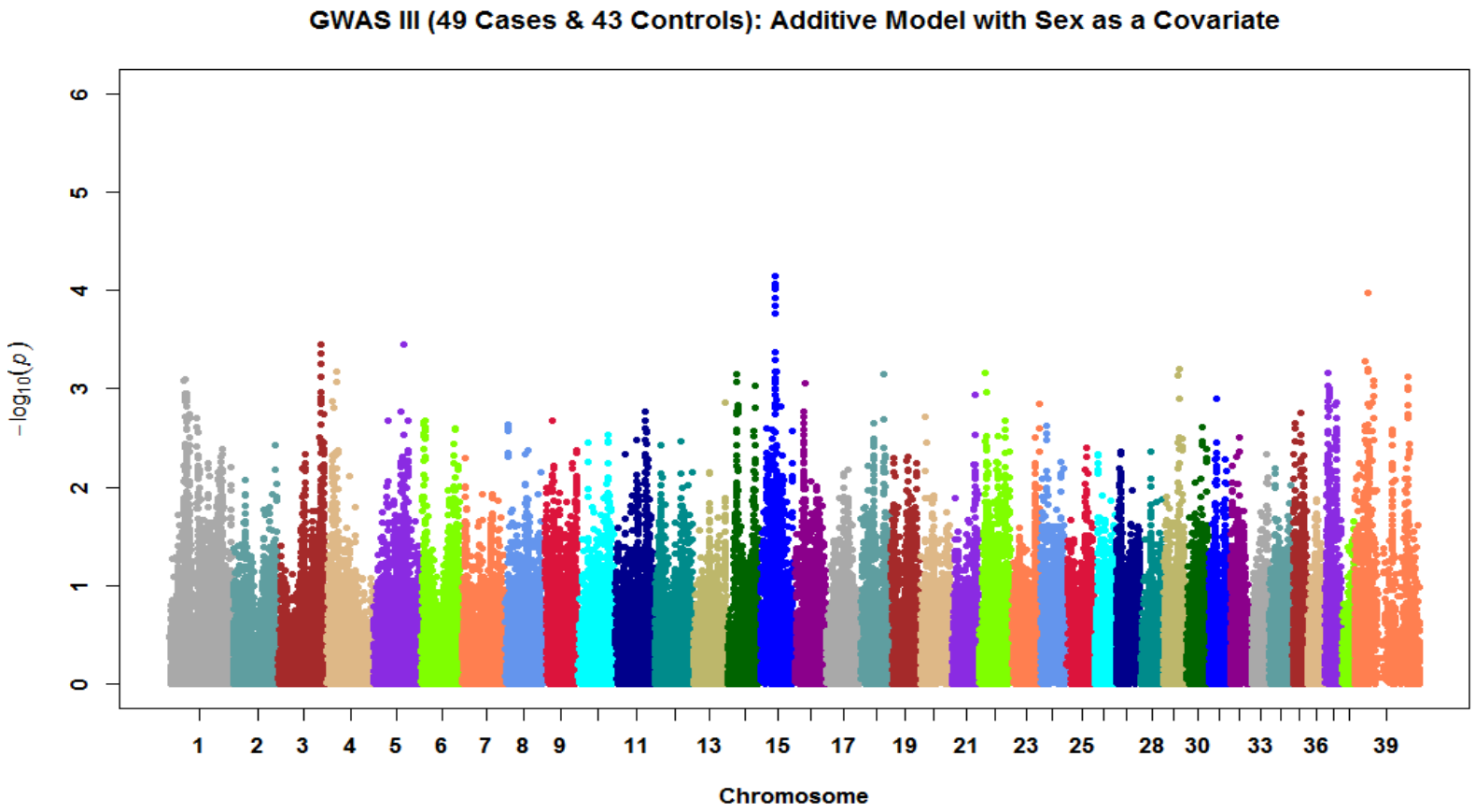



**Figure 14.** Manhattan plot for Miniature Schnauzer GWAS III including 49 dogs with a history of CaOx urolithiasis (cases) and 43 stone-free dogs (controls). The plot displays results of an additive model with sex as a covariate.

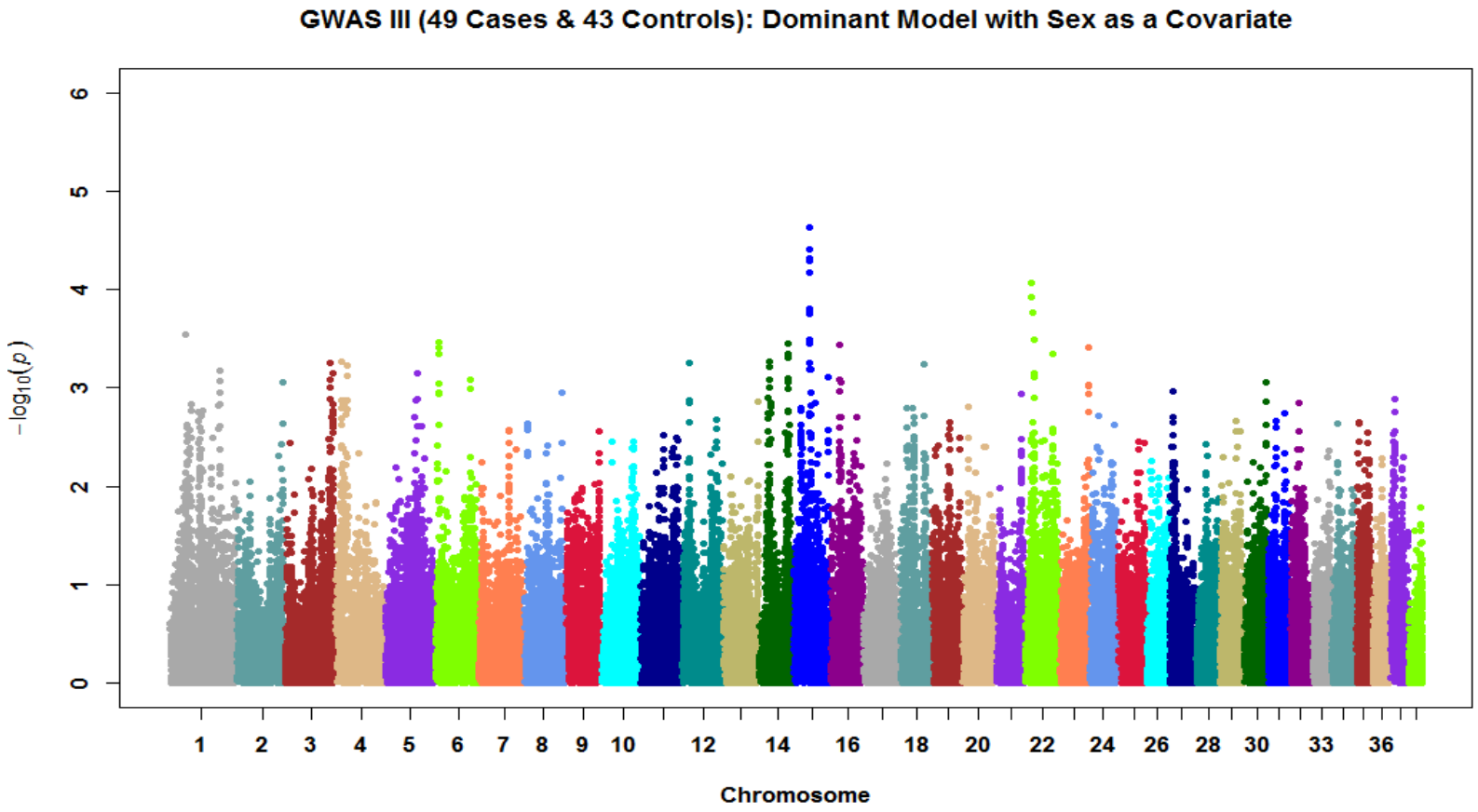



**Figure 15.** Manhattan plot for Miniature Schnauzer GWAS III including 49 dogs with a history of CaOx urolithiasis (cases) and 43 stone-free dogs (controls). The plot displays results of a dominant model with sex as a covariate.

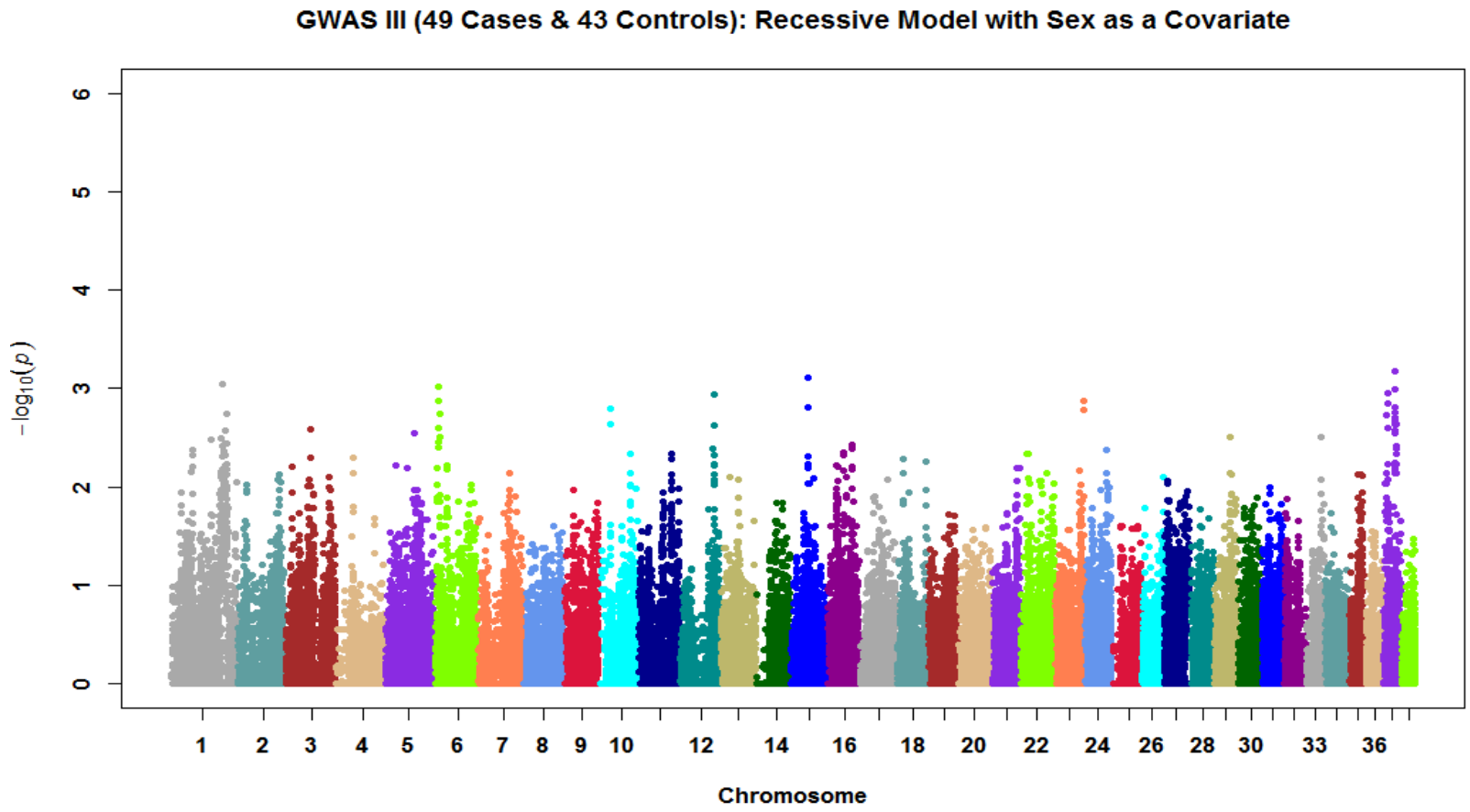



**Figure 16.** Manhattan plot for Miniature Schnauzer GWAS III including 49 dogs with a history of CaOx urolithiasis (cases) and 43 stone-free dogs (controls). The plot displays results of a recessive model with sex as a covariate.

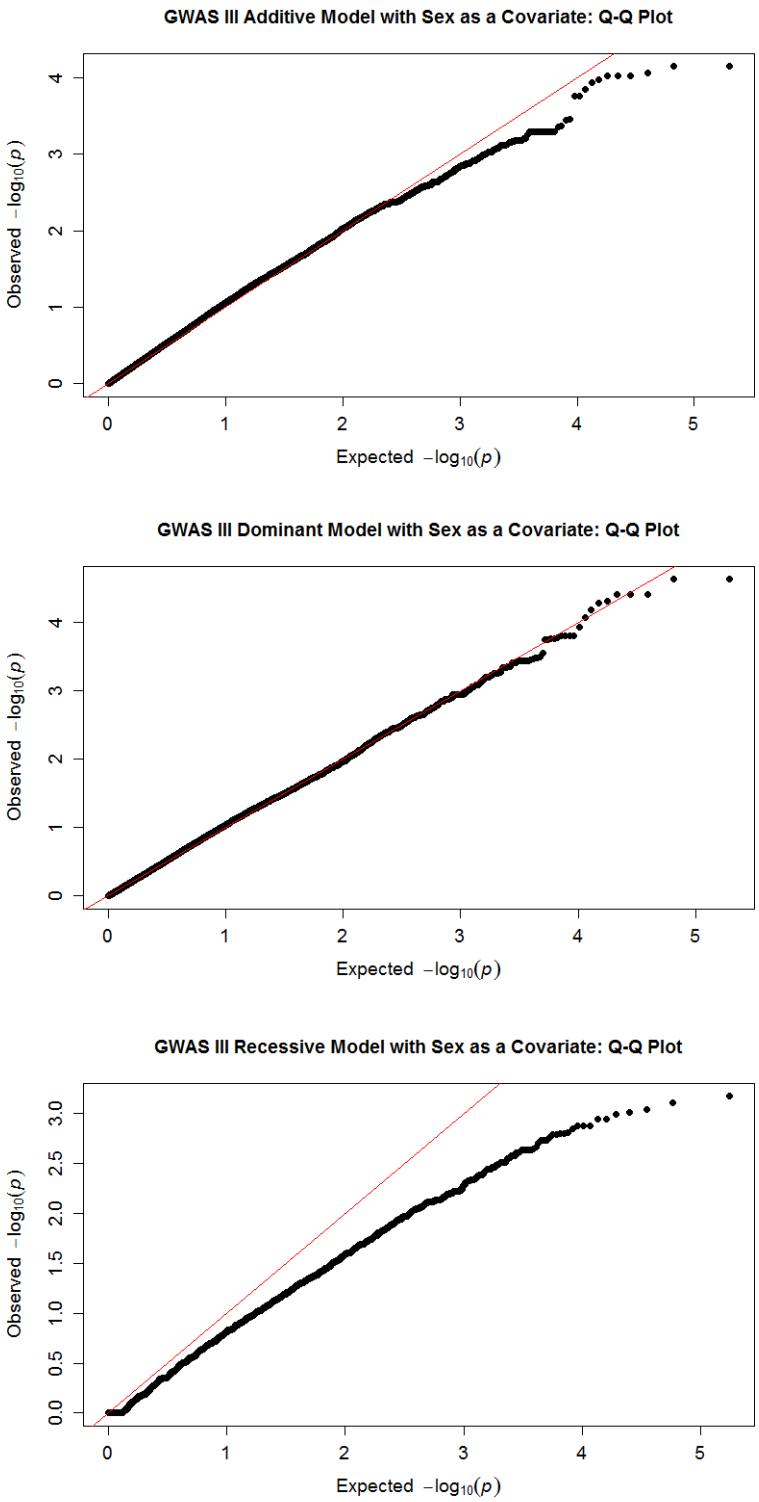



**Figure 17.** Q-Q plots for Miniature Schnauzer GWAS III: A) additive model $GIF_{reg} = 1.03$, B) dominant model $GIF_{reg} = 1.02$, and C) recessive model $GIF_{reg} < 1.00$.

| **Table 8. Bichon Frise CaOx GWAS**<br>**16 Cases (≤ 8 yo, confirmed only) and 10 Controls (≥ 10 yo)** | | | | | |
|---|---|---|---|---|---|
| ***CFA*** | ***SNP*** | ***Position*** | ***OR*** | ***p-value*** | ***p-genome*** |
| **Additive model with sex as a covariate, $GIF_{reg}$ < 1.00, $GIF_{chi}$ = 1.10** | | | | | |
| 15 | BICF2G630429739 | 48180336 | 0.011 | 0.0025 | 0.47 |
| 7 | BICF2G630563403 | 64158000 | 0.018 | 0.0027 | 0.51 |
| 15 | BICF2P752075 | 36071988 | 0.015 | 0.0032 | 0.74 |
| 6 | BICF2P528992 | 39901942 | 0.02 | 0.0032 | 0.74 |
| 6 | BICF2P927925 | 39906412 | 0.02 | 0.0032 | 0.74 |
| 6 | BICF2P1443038 | 39917821 | 0.019 | 0.0032 | 0.74 |
| 6 | BICF2P1230693 | 39921668 | 0.019 | 0.0032 | 0.74 |
| 15 | BICF2S23032337 | 38597342 | 0.019 | 0.0032 | 0.74 |
| 15 | BICF2P1214369 | 46157443 | 0.077 | 0.0032 | 0.75 |
| 7 | BICF2P1369428 | 72568644 | 0.020 | 0.0034 | 0.80 |
| **Dominant model with sex as a covariate, $GIF_{reg}$ < 1.00, $GIF_{chi}$ = 1.04** | | | | | |
| **15** | **BICF2G630431491** | **45744168** | **130** | **0.00097** | **0.046** |
| **15** | **BICF2G630429739** | **48180336** | **0.0076** | **0.00097** | **0.054** |
| 15 | BICF2G630429502 | 48485750 | 0.015 | 0.0017 | 0.36 |
| 6 | BICF2P528992 | 39901942 | 0.016 | 0.0021 | 0.63 |
| 6 | BICF2P927925 | 39906412 | 0.016 | 0.0021 | 0.63 |
| 6 | BICF2P1443038 | 39917821 | 0.016 | 0.0021 | 0.63 |
| 6 | BICF2P1230693 | 39921668 | 0.016 | 0.0021 | 0.63 |
| 6 | BICF2P1429329 | 40711639 | 61 | 0.0021 | 0.62 |
| 6 | BICF2P1470009 | 41154425 | 61 | 0.0021 | 0.62 |
| 6 | BICF2S23125546 | 41802735 | 61 | 0.0021 | 0.62 |
| **Recessive model with sex as a covariate, $GIF_{reg}$ < 1.00, $GIF_{chi}$ < 1.00** | | | | | |
| **15** | **TIGRP2P201486_rs8413090** | **45005683** | **0.013** | **0.0023** | **0.026** |
| 3 | BICF2P41627 | 10731878 | 0.016 | 0.0045 | 0.21 |
| 7 | BICF2P757307 | 36825609 | 0.016 | 0.0045 | 0.22 |
| 7 | BICF2P567109 | 36838754 | 0.016 | 0.0045 | 0.22 |
| 7 | BICF2P538589 | 39505844 | 0.028 | 0.0046 | 0.30 |
| 6 | BICF2S23229527 | 41071588 | 0.026 | 0.0060 | 0.55 |
| 6 | BICF2S22929078 | 41680668 | 0.026 | 0.0060 | 0.55 |
| 3 | BICF2S23314596 | 9374543 | 0.017 | 0.0065 | 0.59 |
| 7 | BICF2P377168 | 35826498 | 0.017 | 0.0065 | 0.61 |
| 7 | BICF2S2348962 | 36890893 | 0.017 | 0.0065 | 0.61 |

SNPs that were met genome-wide significance ($p < 0.05$) are bolded in blue font; those with marginal significance ($p < 0.1$) are in bolded in black font.

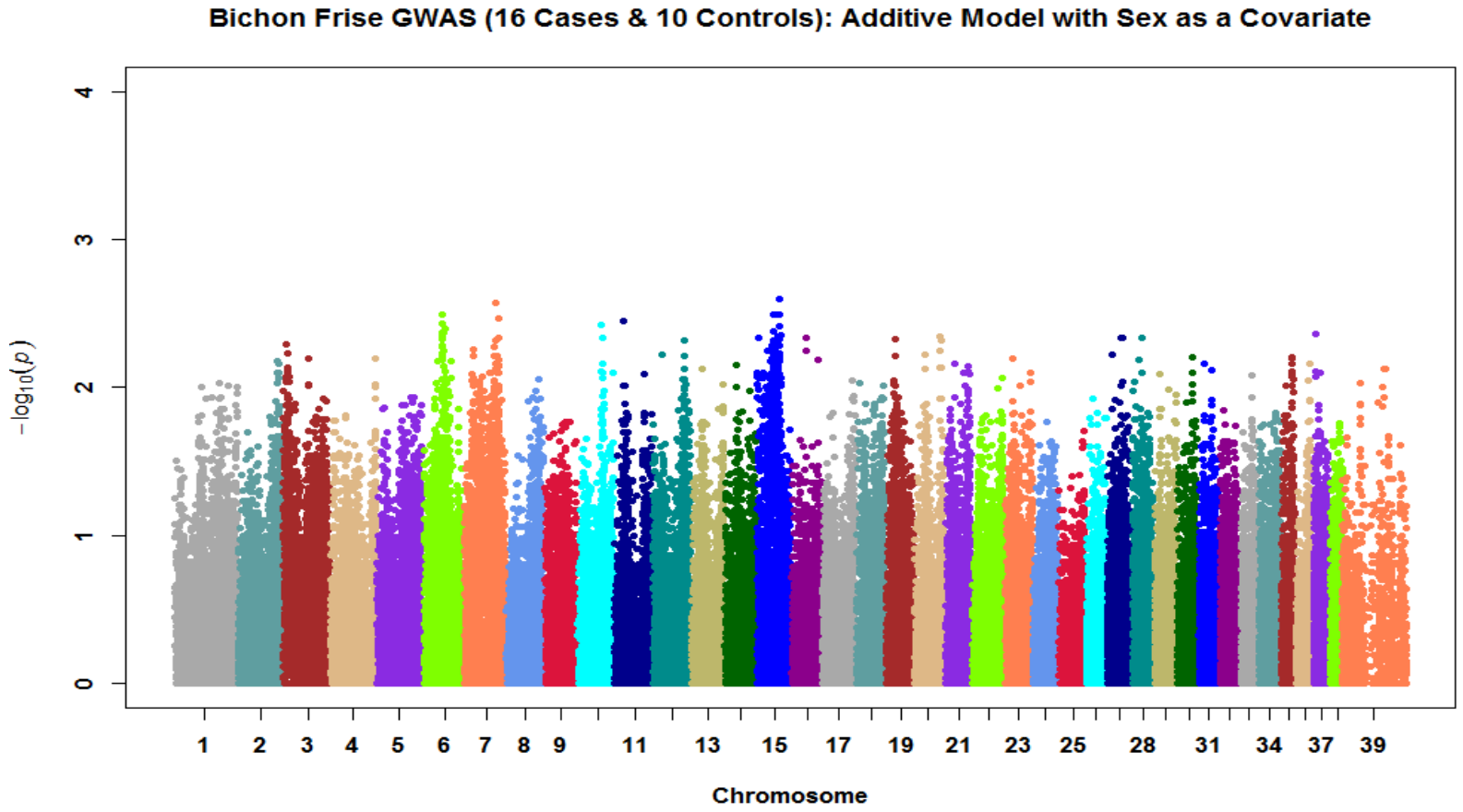



**Figure 18.** Manhattan plot for Bichon Frise GWAS including 16 dogs with a history of CaOx urolithiasis (cases) and 10 stone-free dogs (controls). The plot displays results of an additive model with sex as a covariate.

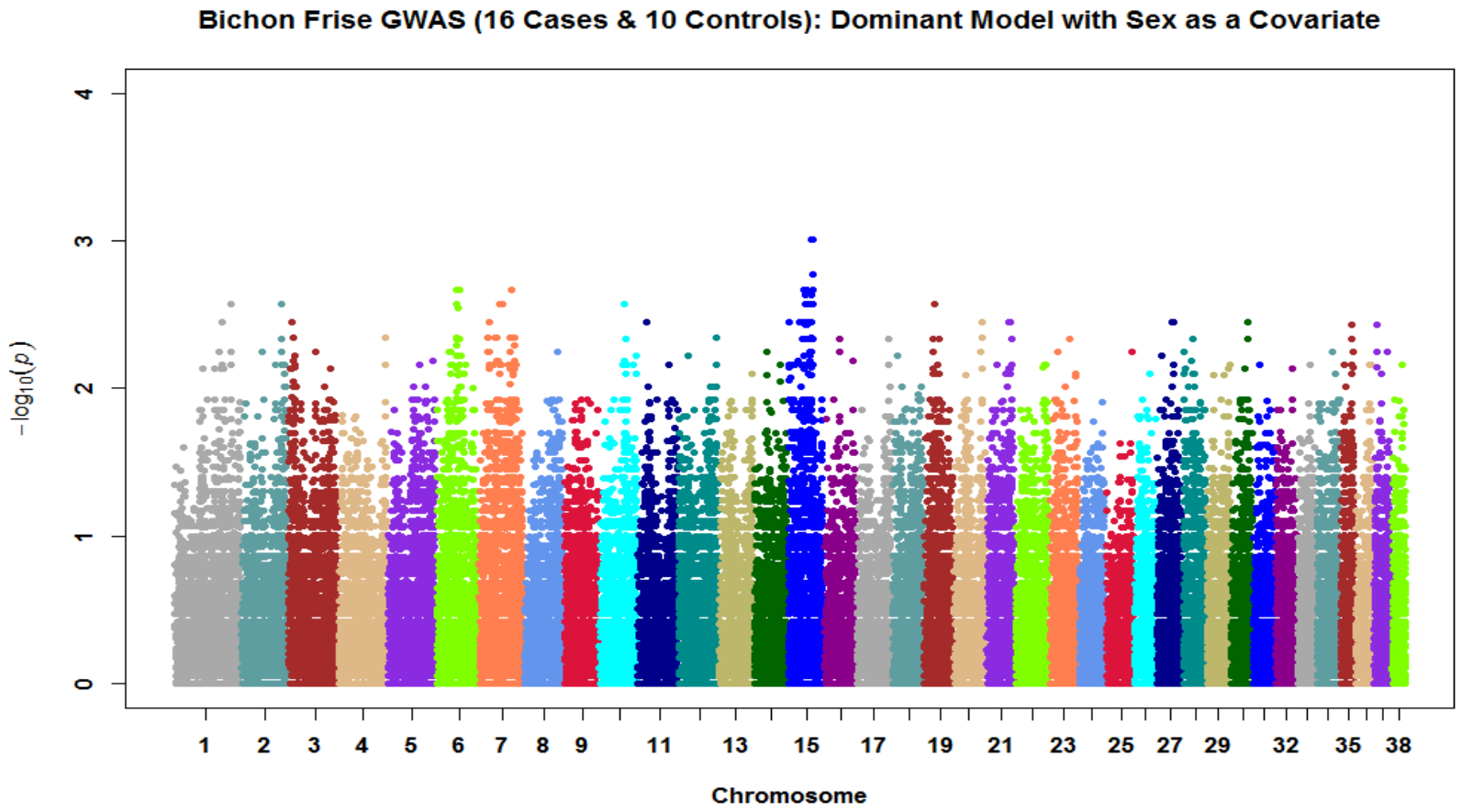



**Figure 19.** Manhattan plot for Bichon Frise GWAS including 16 dogs with a history of CaOx urolithiasis (cases) and 10 stone-free dogs (controls). The plot displays results of a dominant model with sex as a covariate.

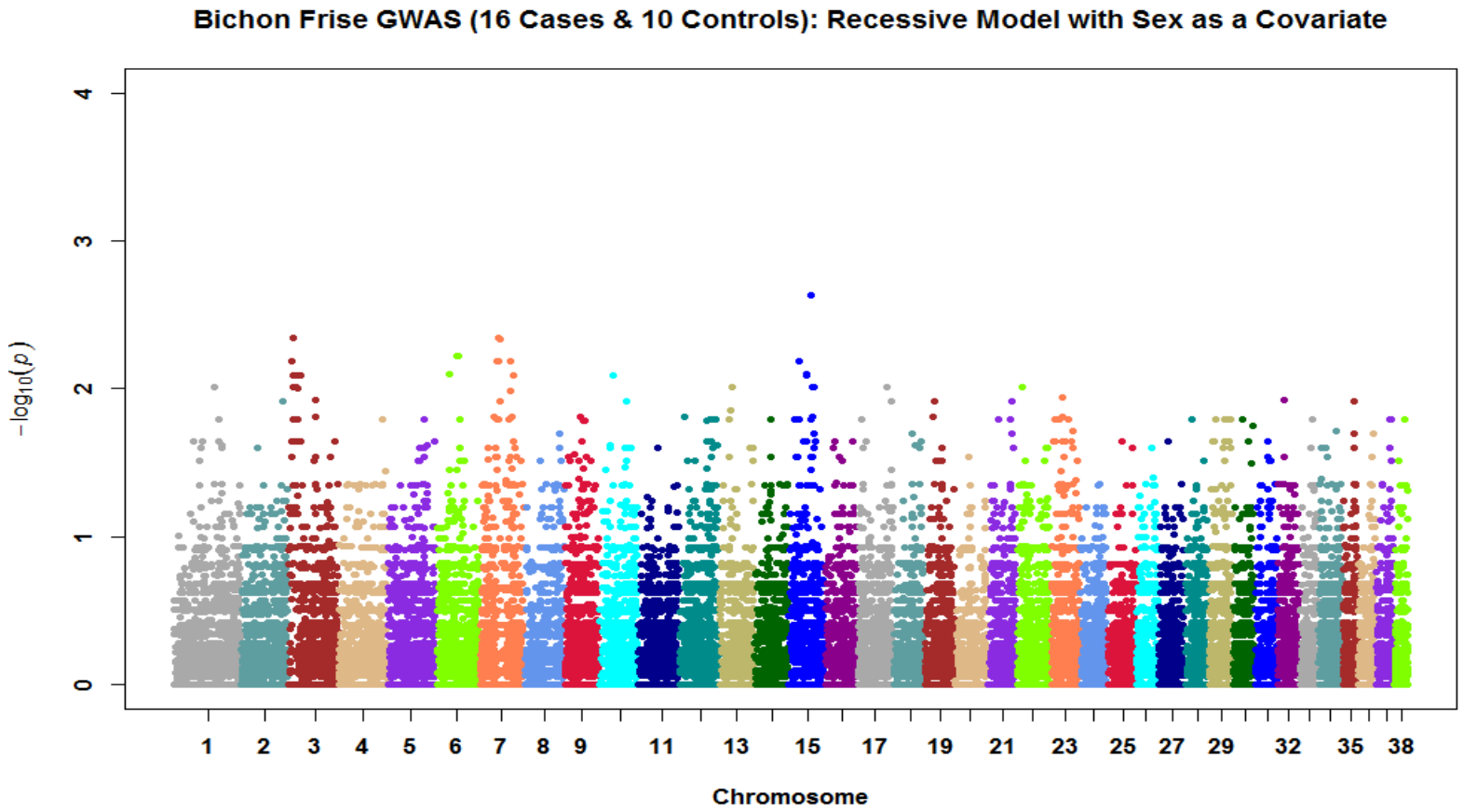
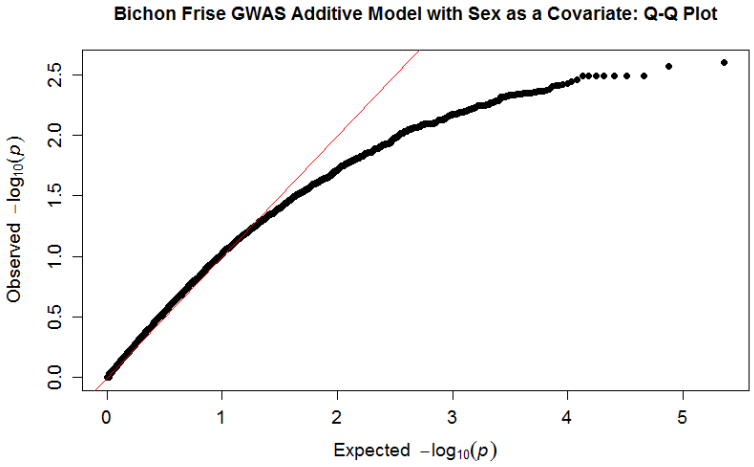
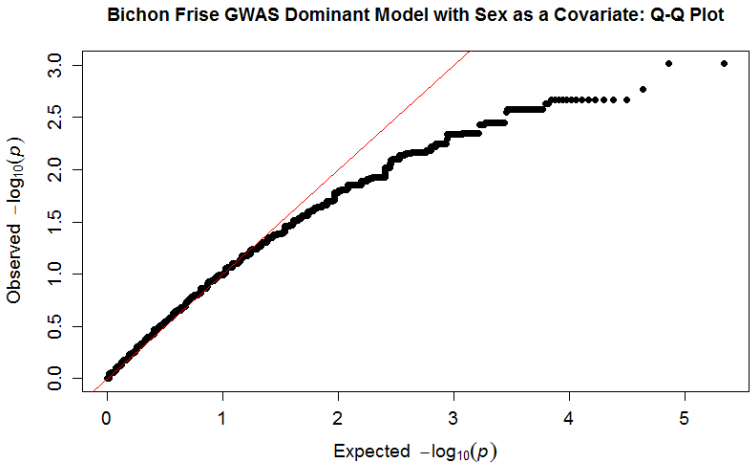



**Figure 20.** Manhattan plot for Bichon Frise GWAS including 16 dogs with a history of CaOx urolithiasis (cases) and 10 stone-free dogs (controls). The plot displays results of a recessive model with sex as a covariate.



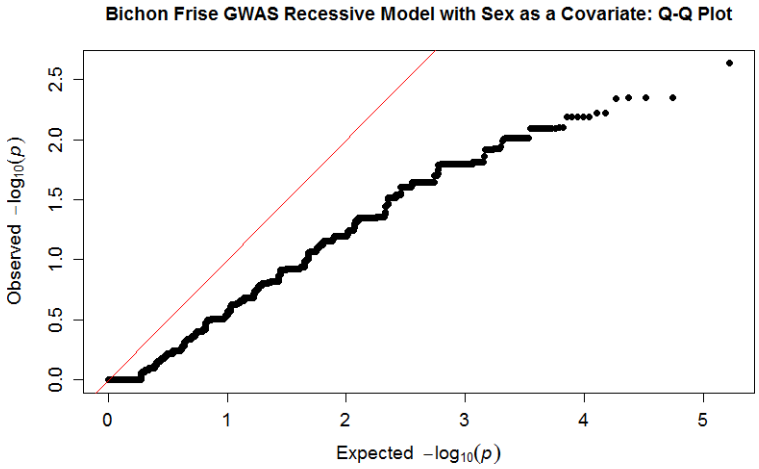




**Figure 21.** Q-Q plots for the Bichon Frise GWAS: A) additive model $GIF_{reg} < 1.00$, B) dominant model $GIF_{reg} < 1.00$, and C) recessive model $GIF_{reg} < 1.00$.

| **Table 9. Bichon Frise CaOx GWAS<br>CFA15 top 5 haplotype blocks (out of 51 total spanning a 10 Mb region)** | | | | | |
|---|---|---|---|---|---|
| ***Block*** | ***Start*** | ***End*** | ***Case, Control Frequency*** | ***OR*** | ***p-value*** |
| 27 | 45469598 | 45496633 | 0.72, 0 | Infinity | 3.8E-7 |
| 11 | 42879417 | 42906756 | 0.94, 0.50 | 15 | 3.0E-4 |
| 16 | 43667654 | 43730706 | 0.59, 0.10 | 13 | 4.0E-4 |
| 41 | 47851215 | 47864748 | 0.97, 0.60 | 21 | 6.0E-4 |
| 37 | 46823335 | 46829000 | 0.81, 0.35 | 8.0 | 8.0E-4 |

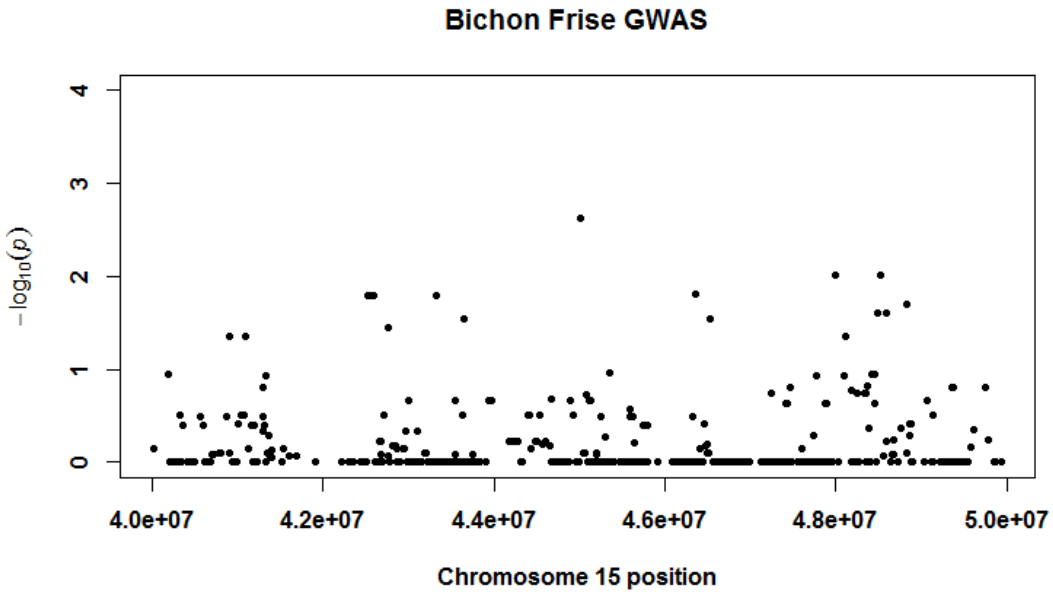



**Figure 22.** Results for a 10 Mb region on CFA15 that encompasses the top SNPs associated with CaOx urolithiasis in Bichon Frise GWAS.

| **Table 10. Bichon Frise CaOx GWAS & Multibreed CaOx GWAS (region 1)<br>Protein-coding genes in the top haplotype containing region of CFA15<br>g.42879417 – 47864748 bp** | | | | | |
|---|---|---|---|---|---|
| *ANO4* | ***SLC5A8*** | *UTP20* | *ARL1* | *SPIC* | *MYBPC1* |
| *CHPT1* | *GNPTAB* | *DRAM1* | *CCDC53* | *NUP37* | *PARPBP* |
| *PMCH* | *IGF1* | *PAH* | *ASCL1* | *STAB2* | *NT5DC3* |
| *HSP90B1* | *C12ORF73* | *TDG* | *GLT8D2* | *HCFC2* | *NFYB* |
| *SMARCA5* | *GYPA* | *HHIP* | *ANAPC10* | *ABCE1* | *OTUD4* |
| *SMAD1* | *MMAA* | *ZNF827* | *YWHAZ* | *LSM6* | ***SLC10A7*** |

Top candidate genes based on function and tissue localization are bolded in blue font.

| **Table 11. Multibreed (Miniature Schnauzer and Bichon Frise) CaOx GWAS 65 Cases (≤ 8 yo, confirmed only) and 53 Controls (≥ 10 yo)** | | | | | |
|---|---|---|---|---|---|
| ***CFA*** | ***SNP*** | ***Position*** | ***OR*** | ***p-value*** | ***p-genome*** |
| **Cochran-Mantel-Haenszel with sex as a covariate, $GIF_{reg}$ = 1.18, $GIF_{chi}$ = 1.16** | | | | | |
| **15** | **BICF2G630432929** | **33089022** | **0.21** | **8.7E-08** | **0.0050** |
| **15** | **BICF2G630433365** | **32523939** | **0.24** | **6.4E-07** | **0.032** |
| **15** | **BICF2G630433434** | **32366495** | **0.26** | **8.8E-07** | **0.049** |
| **15** | **BICF2G630433416** | **32429846** | **0.26** | **8.8E-07** | **0.049** |
| **15** | **BICF2P270746** | **32468068** | **4.2** | **1.5E-06** | **0.078** |
| 15 | BICF2G630433352 | 32534722 | 4.1 | 2.2E-06 | 0.12 |
| 15 | BICF2G630433442 | 32320032 | 0.27 | 2.4E-06 | 0.13 |
| 15 | BICF2G630433424 | 32383555 | 3.9 | 3.7E-06 | 0.19 |
| 15 | TIGRP2P201105_rs8678457 | 43667654 | 6.8 | 3.7E-06 | 0.19 |
| 15 | BICF2S23343598 | 43680121 | 6.8 | 3.7E-06 | 0.19 |

SNPs that were met genome-wide significance ($p < 0.05$) are bolded in blue font; those with marginal significance ($p < 0.1$) are in bolded in black font.

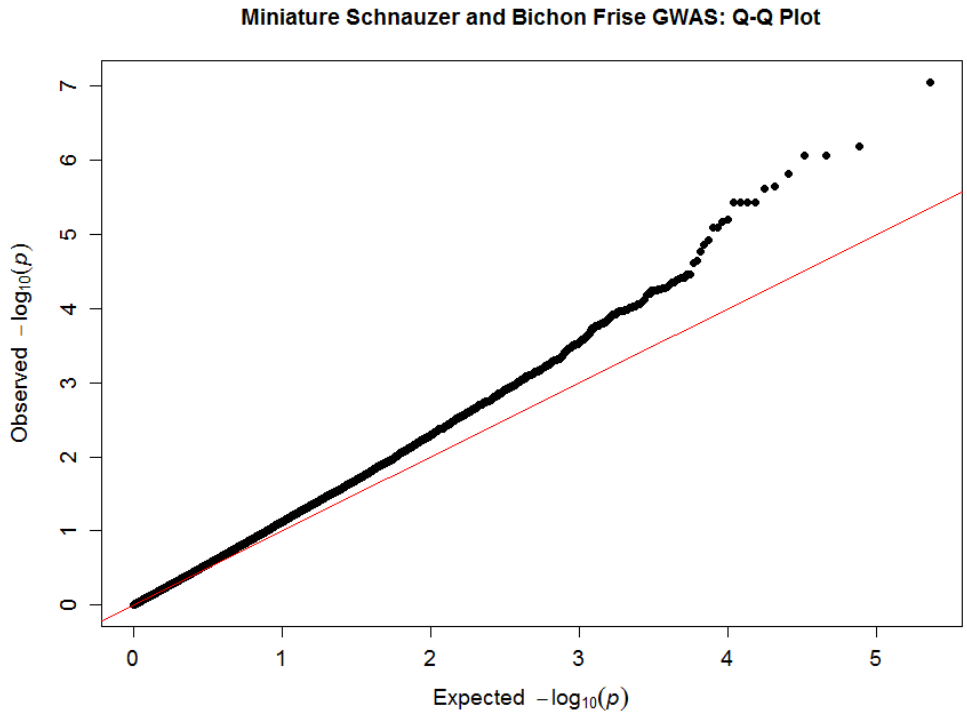



**Figure 23.** Q-Q plot for the multibreed GWAS using a Cochran-Mantel-Haenszel test with sex as a covariate, $GIF_{reg}$ = 1.18.

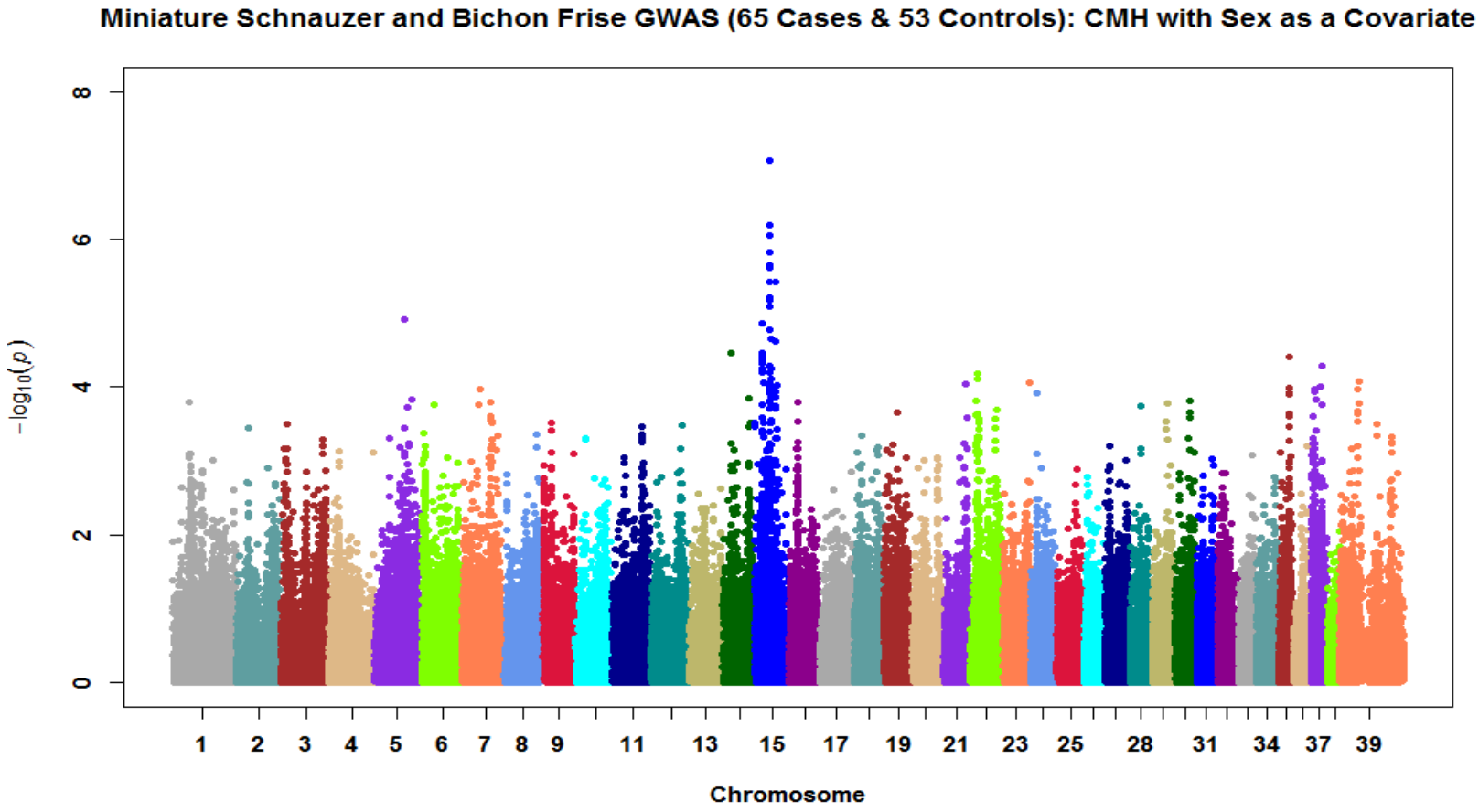



**Figure 24.** Manhattan plot for the multibreed GWAS including 65 dogs with a history of CaOx urolithiasis (cases) and 53 stone-free dogs (controls). The plot displays results of the Cochran-Mantel-Haenszel test with sex as a covariate.

| Table 12. Multibreed (Miniature Schnauzer and Bichon Frise) GWAS GenABEL mixed model with the 1st PCA and sex as covariates, GIF = 1.06 | | | | | |
|---|---|---|---|---|---|
| ***CFA*** | ***SNP*** | ***Position*** | ***B allele effect*** | ***p-value*** | ***p-genome*** |
| **15** | **BICF2G630432929** | **33089022** | **-0.35** | **1.6E-07** | **0.004** |
| **15** | **BICF2G630433434** | **32366495** | **-0.29** | **1.8E-06** | **0.045** |
| **15** | **BICF2G630433416** | **32429846** | **-0.29** | **1.8E-06** | **0.045** |
| 15 | BICF2G630433442 | 32320032 | -0.29 | 4.0E-06 | 0.13 |
| 15 | BICF2G630433365 | 32523939 | -0.29 | 5.0E-06 | 0.16 |
| 15 | BICF2P270746 | 32468068 | 0.29 | 5.0E-06 | 0.13 |
| 15 | BICF2G630433352 | 32534722 | 0.29 | 8.0E-06 | 0.19 |
| 15 | TIGRP2P201105_rs8678457 | 43667654 | 0.35 | 9.8E-06 | 0.59 |
| 15 | BICF2S23343598 | 43680121 | 0.35 | 9.8E-06 | 0.59 |
| 15 | TIGRP2P201118_rs8993300 | 43701090 | 0.35 | 9.8E-06 | 0.59 |

SNPs that were met genome-wide significance ($p < 0.05$) are bolded in blue font; those with marginal significance ($p < 0.1$) are in bolded in black font.

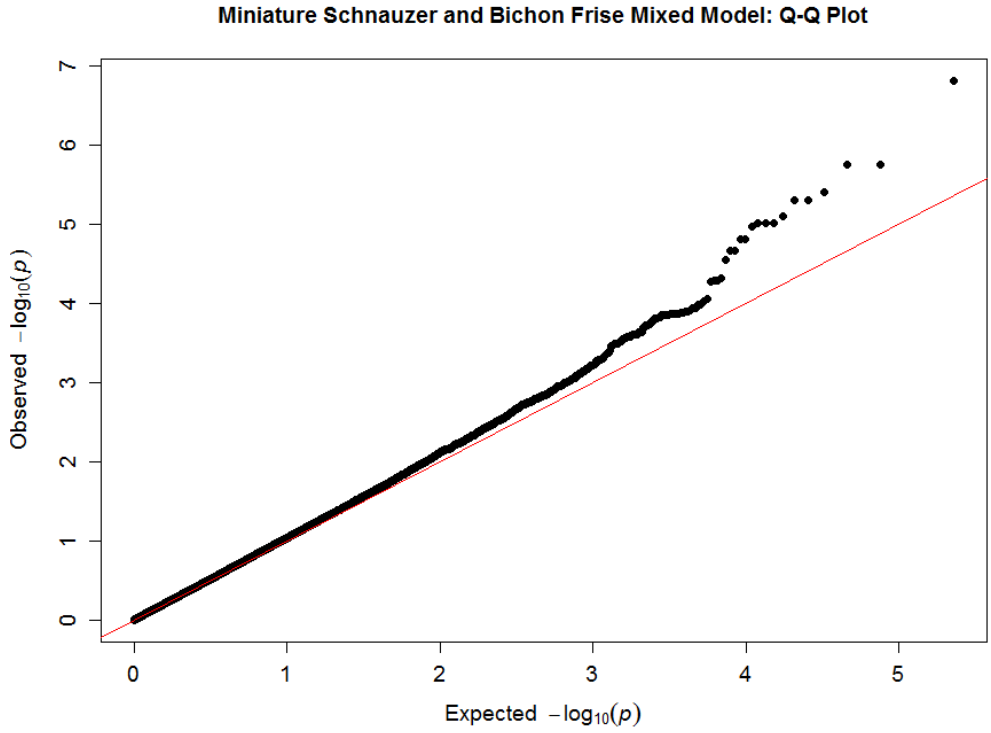



**Figure 25.** Q-Q plot for the multibreed GWAS using a mixed model analysis with the 1st principal component and sex as covariates, GIF = 1.06.

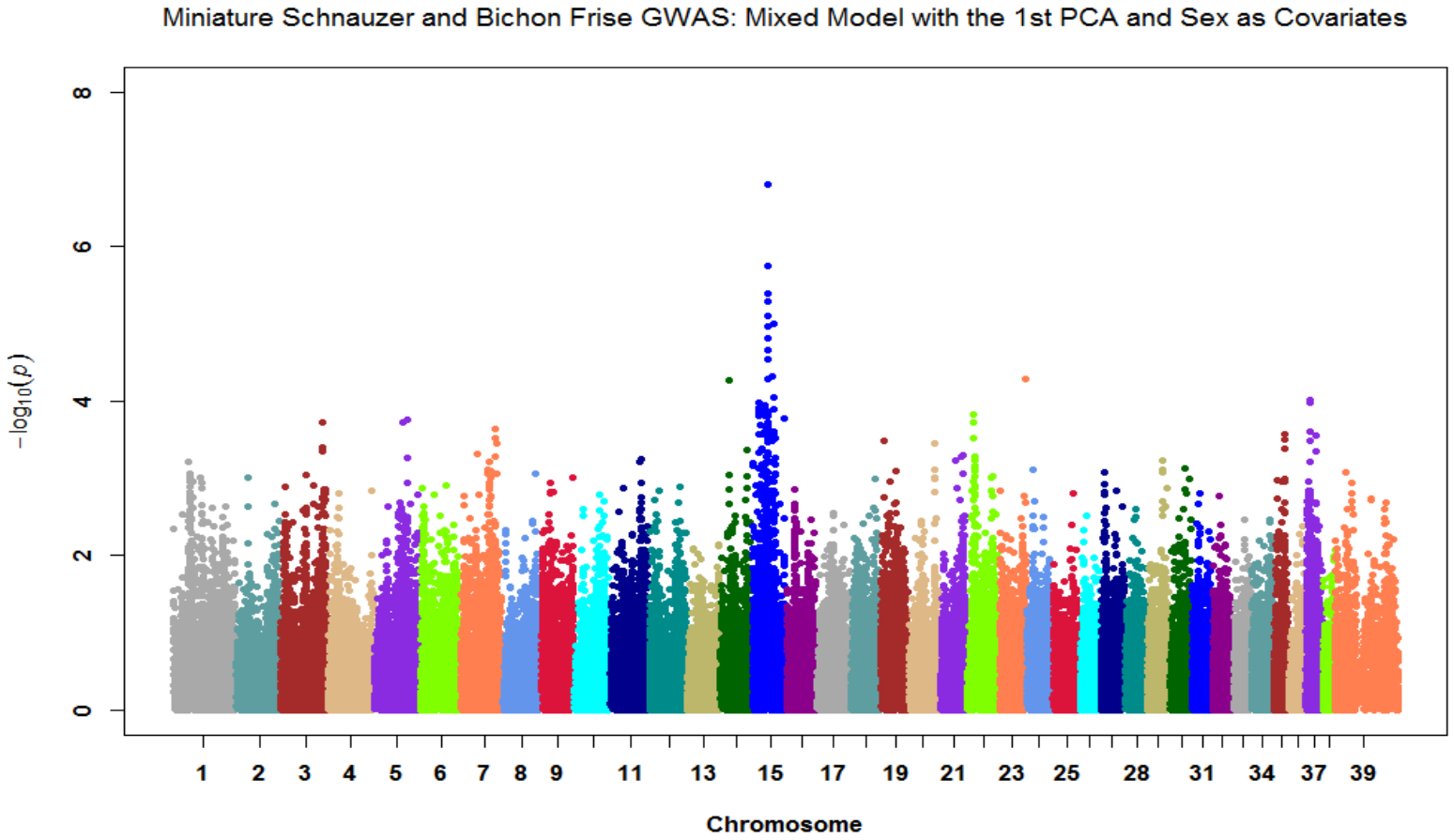



**Figure 26.** Manhattan plot for the multibreed GWAS including 65 dogs with a history of CaOx urolithiasis (cases) and 53 stone-free dogs (controls). The plot displays results of a mixed model with the 1st principal component and sex as covariates.

| **Table 13. Multibreed (Miniature Schnauzer and Bichon Frise) CaOx GWAS CFA15 top 10 haplotype blocks (out of 168 total spanning a 20 Mb region)** | | | | | | |
|---|---|---|---|---|---|---|
| ***Block*** | ***Multibreed Region*** | ***Start*** | ***End*** | ***Case, Control Frequency*** | ***OR*** | ***p-value*** |
| 134 | 1 | 45414471 | 45496633 | 0.19, 0.01 | 24 | 1.4E-05 |
| 141 | 1 | 46084404 | 46095799 | 0.15, 0 | Infinity | 4.3E-05 |
| 119 | 1 | 43667654 | 43701090 | 0.25, 0.06 | 5.7 | 5.2E-05 |
| 36 | 2 | 32320032 | 32627303 | 0.55, 0.30 | 2.8 | 0.00020 |
| 117 | 1 | 43321939 | 43389465 | 0.19, 0.03 | 7.8 | 0.00020 |
| 157 | 1 | 47543364 | 47553640 | 0.16, 0.02 | 10 | 0.00020 |
| 118 | 1 | 43581114 | 43610768 | 0.28, 0.03 | 7.4 | 0.00030 |
| 37 | 2 | 32650383 | 32743747 | 0.71, 0.48 | 2.6 | 0.00030 |
| 46 | 2 | 33571267 | 33584438 | 0.56, 0.33 | 2.6 | 0.00040 |
| 41 | 2 | 33122175 | 33188556 | 0.36, 0.16 | 3.0 | 0.00050 |

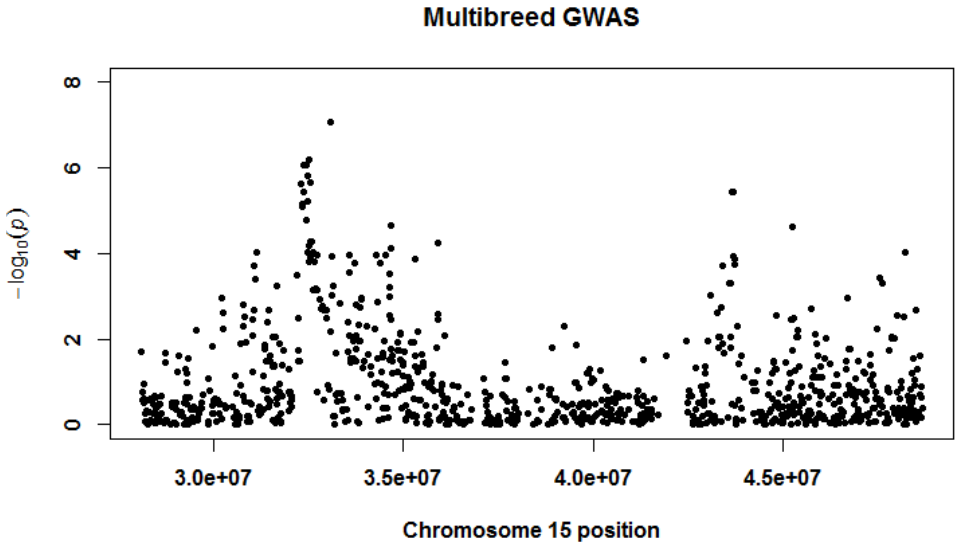



**Figure 27.** Results for a 20 Mb region on CFA15 that encompasses the top SNPs associated with CaOx urolithiasis in the multibreed GWAS.

| **Table 14. Multibreed (Miniature Schnauzer and Bichon Frise) CaOx GWAS Protein-coding genes in the top haplotype containing region 2 of CFA15 32,320,032 – 33,584,438** | | | | | |
|---|---|---|---|---|---|
| *TMTC3* | *KITLG* | *DUSP6* | *POC1B* | *GALNT4* | ***ATP2B1*** |

Top candidate gene based on function and tissue localization is bolded in blue font.

| **Table 15. Miniature Schnauzer CaOx GWAS III reanalyzed with CFA15 region 1 haplotype<br>49 Cases (≤ 8 yo, confirmed only) and 43 Controls (≥ 10 yo)** | | | | | |
|---|---|---|---|---|---|
| ***CFA*** | ***SNP*** | ***Position*** | ***OR*** | ***p-value*** | ***p-genome*** |
| **Additive model with CFA15 and sex as covariates, $GIF_{reg}$ = 1.00, $GIF_{chi}$ = 1.07** | | | | | |
| 22 | BICF2G630318063 | 14088828 | 0.13 | 9.8E-05 | 0.33 |
| 37 | TIGRP2P423581_rs8897643 | 8736596 | 5.2 | 0.00014 | 0.45 |
| 39 | BICF2P131020 | 24776612 | 33 | 0.00014 | 0.47 |
| 15 | BICF2G630433325 | 32567886 | 0.23 | 0.00016 | 0.53 |
| 15 | BICF2G630433322 | 32570491 | 0.23 | 0.00016 | 0.53 |
| 15 | BICF2G630433396 | 32498694 | 0.24 | 0.00022 | 0.67 |
| 15 | BICF2G630433386 | 32503260 | 0.24 | 0.00022 | 0.67 |
| 15 | BICF2G630433332 | 32546804 | 0.22 | 0.00022 | 0.68 |
| 15 | BICF2G630433404 | 32482976 | 0.24 | 0.00022 | 0.68 |
| 37 | BICF2S23712480 | 8742264 | 4.4 | 0.00025 | 0.73 |
| **Dominant model with CFA15 and sex as a covariate, $GIF_{reg}$ = 1.00, $GIF_{chi}$ =1.00** | | | | | |
| **15** | **BICF2G630433325** | **32567886** | **0.067** | **2.0E-05** | **0.10** |
| **15** | **BICF2G630433322** | **32570491** | **0.067** | **2.0E-05** | **0.10** |
| 22 | BICF2G630318063 | 14088828 | 0.083 | 3.0E-05 | 0.16 |
| 15 | BICF2G630433404 | 32482976 | 0.072 | 3.5E-05 | 0.20 |
| 15 | BICF2G630433396 | 32498694 | 0.074 | 3.8E-05 | 0.21 |
| 15 | BICF2G630433386 | 32503260 | 0.074 | 3.8E-05 | 0.21 |
| 15 | BICF2G630433373 | 32513694 | 0.073 | 4.8E-05 | 0.27 |
| 22 | BICF2G630318057 | 14086646 | 0.11 | 5.1E-05 | 0.28 |
| 15 | BICF2G630433365 | 32523939 | 0.078 | 5.2E-05 | 0.29 |
| 15 | BICF2G630433332 | 32546804 | 0.064 | 7.9E-05 | 0.48 |
| **Recessive model with CFA15 and sex as a covariate, $GIF_{reg}$ <1.00, $GIF_{chi}$ < 1.00** | | | | | |
| 37 | BICF2P304758 | 8637024 | 9.5 | 0.00039 | 0.19 |
| 37 | TIGRP2P423581_rs8897643 | 8736596 | 18 | 0.00040 | 0.20 |
| 37 | BICF2S23712480 | 8742264 | 11 | 0.00047 | 0.26 |
| 37 | TIGRP2P420378_rs8991216 | 24538218 | 0.079 | 0.00051 | 0.30 |
| 37 | BICF2G630126438 | 9750129 | 27 | 0.00060 | 0.39 |
| 15 | BICF2G630432586 | 33571267 | 10 | 0.00076 | 0.53 |
| 15 | BICF2G630432577 | 33584438 | 10 | 0.00076 | 0.53 |
| 37 | TIGRP2P420339_rs8525661 | 24455958 | 0.085 | 0.00077 | 0.54 |
| 6 | G378f45S224 | 10006580 | 15 | 0.00087 | 0.62 |
| 37 | BICF2P457642 | 23741393 | 0.095 | 0.0011 | 0.74 |

SNPs with marginal significance ($p < 0.1$) at the genome-wide level are bolded.

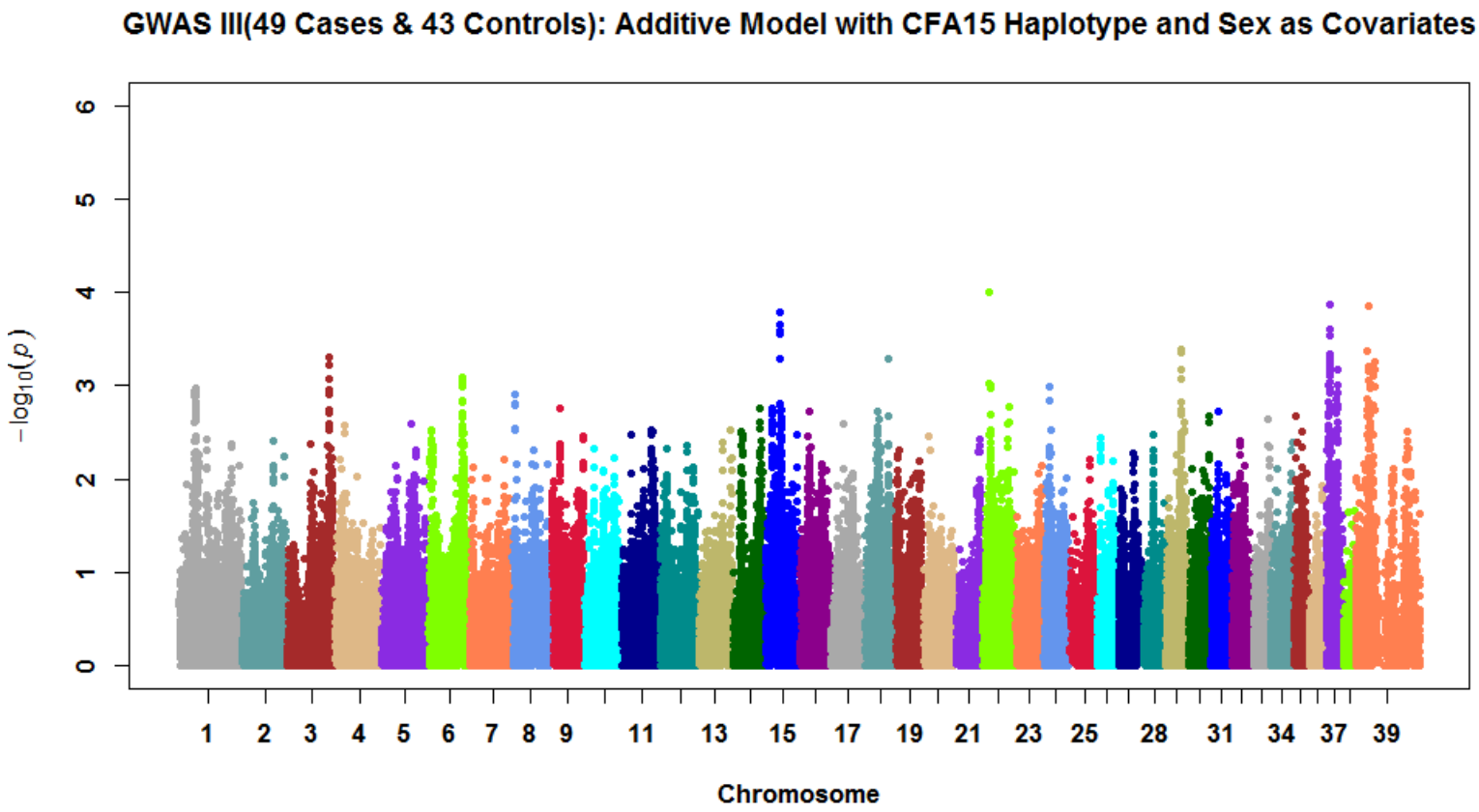



**Figure 28.** Manhattan plot for Miniature Schnauzer GWAS including 49 dogs with a history of CaOx urolithiasis (cases) and 43 stone-free dogs (controls). The plot displays results of an additive model with the multibreed CFA15 region 1 risk haplotype and sex as covariates.

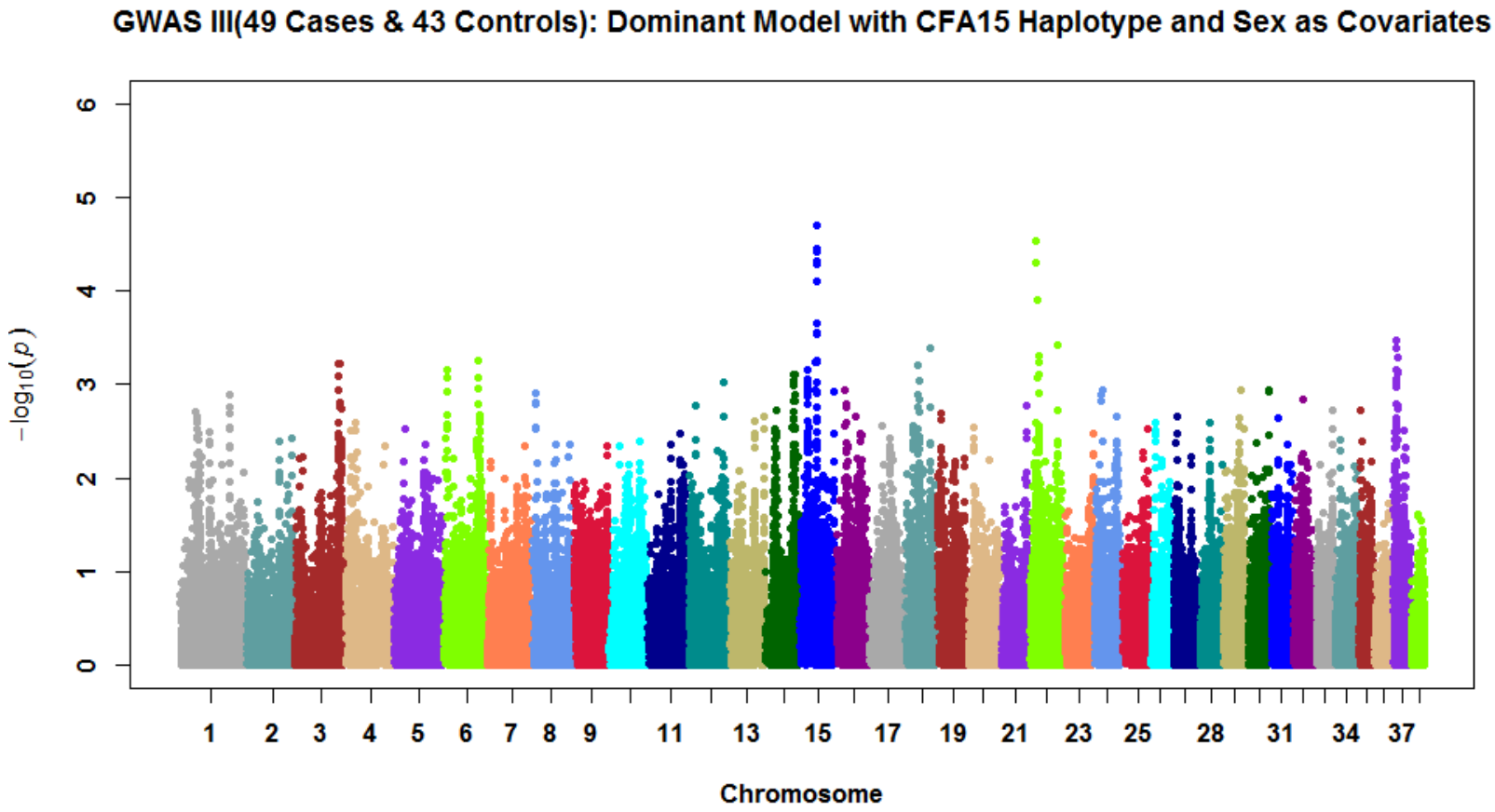



**Figure 29.** Manhattan plot for Miniature Schnauzer GWAS including 49 dogs with a history of CaOx urolithiasis (cases) and 43 stone-free dogs (controls). The plot displays results of a dominant model with the multibreed CFA15 region 1 risk haplotype and sex as covariates.

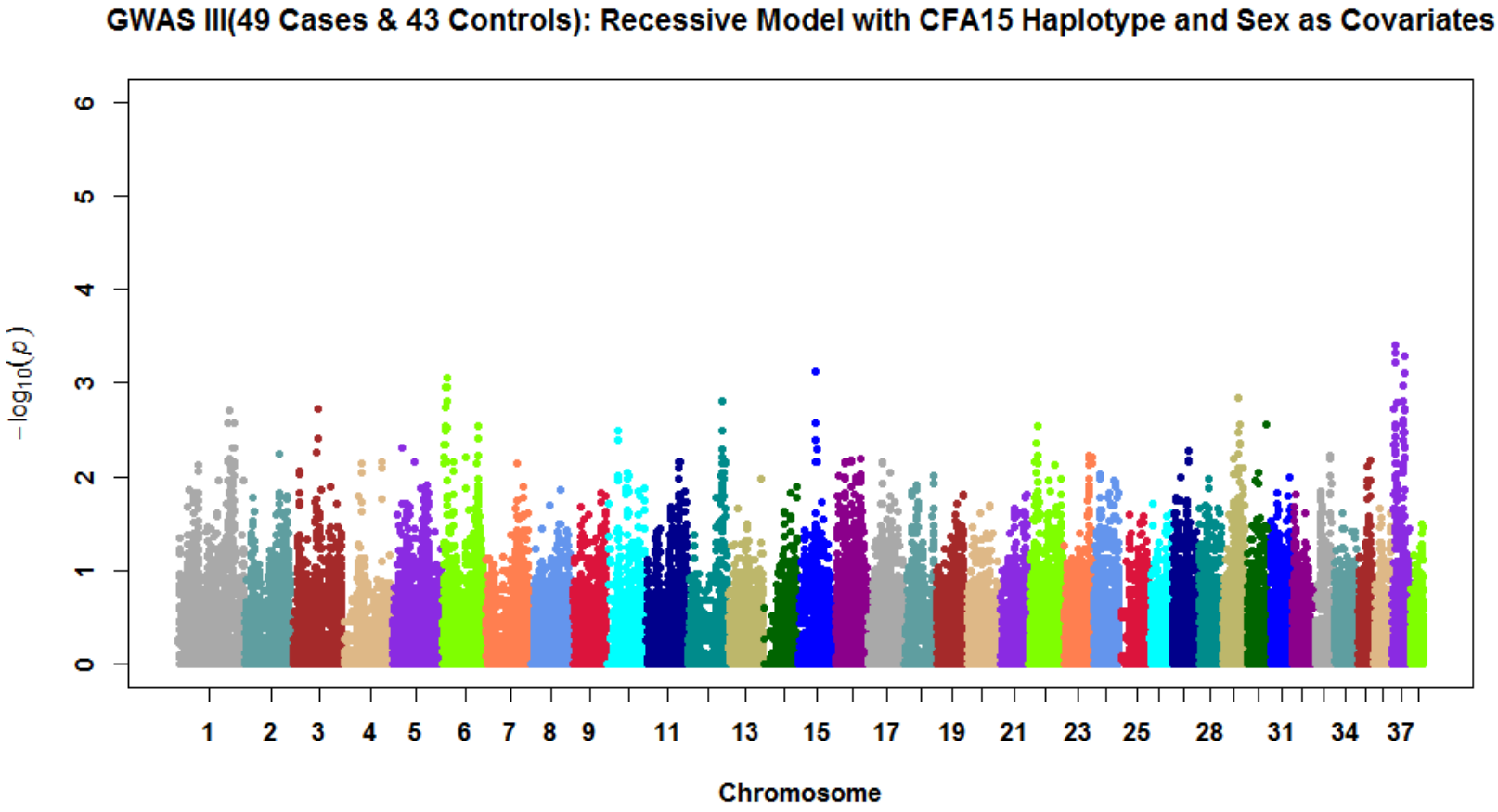



**Figure 30.** Manhattan plot for Miniature Schnauzer GWAS including 49 dogs with a history of CaOx urolithiasis (cases) and 43 stone-free dogs (controls). The plot displays results of a recessive model with the multibreed CFA15 region 1 risk haplotype and sex as covariates.

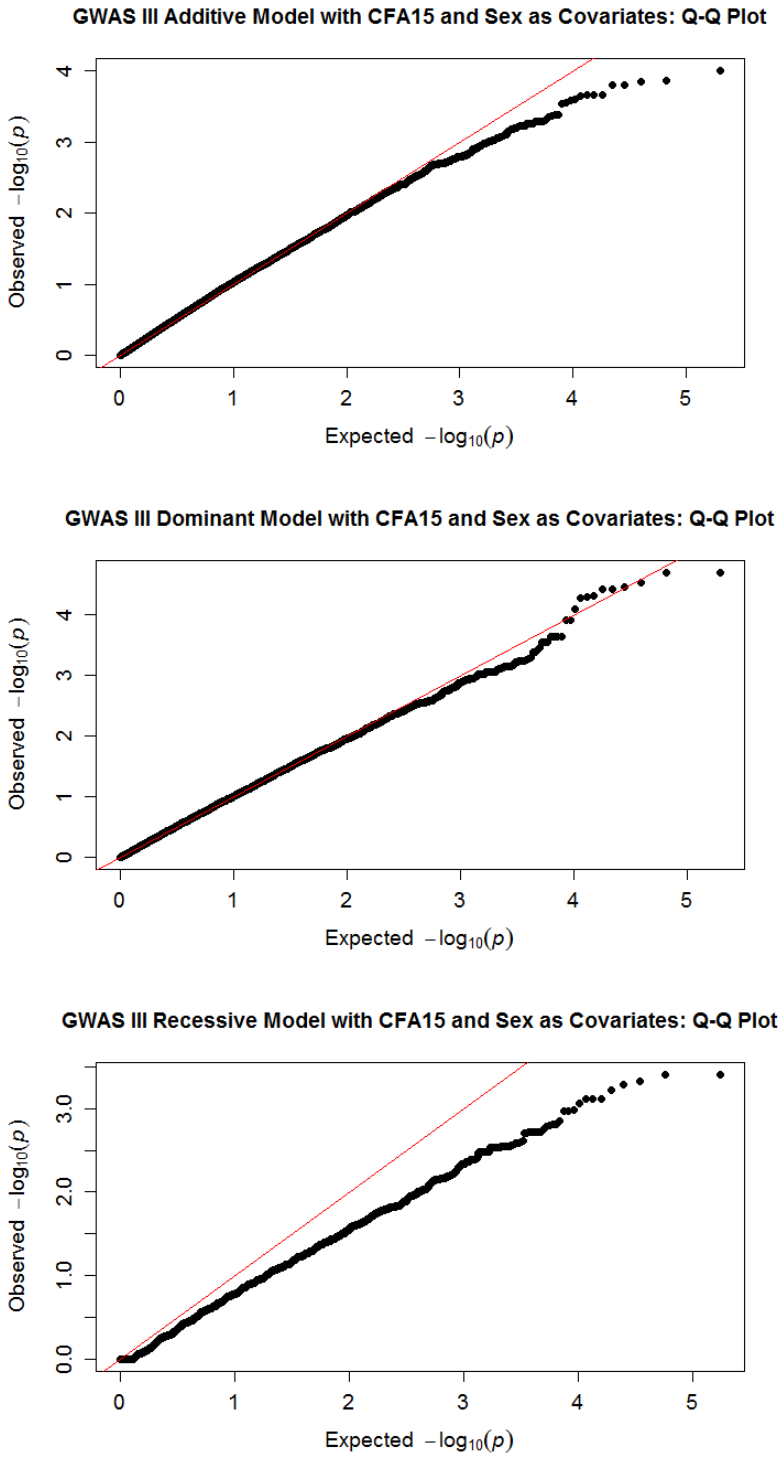



**Figure 31.** Q-Q plots for Miniature Schnauzer GWAS III with both CFA15 region 1 risk haplotype and sex as covariates: A) additive model $GIF_{reg} = 1.00$, B) dominant model $GIF_{reg} = 1.00$, and C) recessive model $GIF_{reg} < 1.00$.

# CHAPTER 5

## Resequencing of a canine chromosome 37 locus associated with calcium oxalate urolithiasis in Miniature Schnauzer dogs

**Resequencing of a canine chromosome 37 locus associated with calcium oxalate urolithiasis in Miniature Schnauzer dogs**

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## Summary

We previously identified a susceptibility locus for CaOx urolithiasis on CFA37 in Miniature Schnauzer dogs. The aim of this study was to perform whole-genome re-sequencing of two CaOx case and two stone-free control Miniature Schnauzer dogs to uncover a putative causal mutation at the CFA37 risk locus. We hypothesized that a mutation would be found within the top positional candidate gene, *SLC39A10*. Coding variants were detected in 3/18 genes in the region, but these variants were considered likely benign due to poor conservation scores within vertebrates and/or no predicted effect on protein sequence. No coding variants were present in *SLC39A10*, but there were two putative non-coding regulatory mutations in the case dogs (a 16 bp 3'UTR duplication and a 23 bp intronic deletion). However, analysis of these two variants in a large cohort (174 Miniature Schnauzers) revealed that they had a weak association with stone status relative to other regional markers. Though a causal mutation was not discovered in this analysis, sequence coverage was missing for the highly conserved *SLC39A10* promoter and for a coding exon in another positional candidate gene. Future studies must be performed to obtain and analyze the missing sequence and further evaluate for regulatory variants at the CFA37 CaOx-associated locus.

## Introduction

Nephrolithiasis is a common and expensive health problem that costs the United States an estimated 5 billion dollars annually (4). More than half of the risk for kidney stones is estimated to be inherited (19), but susceptibility genes have largely evaded discovery. Dogs offer a spontaneous model of calcium oxalate (CaOx) urolithiasis, the most common kidney stone type affecting people (9). Importantly, selective breeding and population bottlenecks have resulted in within-breed population structures that are highly amenable to genetic studies (62).

We previously performed a genome-wide association study (GWAS) for CaOx urolithiasis in Miniature Schnauzer dogs and identified a region of CFA37 that is strongly associated with stone risk (**Chapter 4**). The region spans 2 Mb and contains 18 protein-coding genes. One of the genes in this region is a solute carrier gene, *SLC39A10.* Solute carrier genes are commonly implicated in stone disease. Mutations in *SLC2A9* impart risk for urate stones in dogs and people (71-73), mutations in *SLC3A1* and *SLC7A9* cause cystine stones in both species (74-76), and several of the rare monogenic disorders associated with hypercalciuria and CaOx urolithiasis in people are caused by solute carrier mutations (26-29, 37).

The aim of this study was to perform whole-genome re-sequencing (WGS) of Miniature Schnauzer dogs with variant discovery for the genes residing within the CFA37 risk locus. Coding sequence was examined for all 18 genes at the CFA37 locus, and both coding sequence and regulatory regions were evaluated for *SLC39A10*. Our hypothesis was that *SLC39A10* harbors a causal mutation for CaOx urolithiasis risk in Miniature Schnauzers.

## Materials and Methods

*Ethics statement and sample collection*

The study protocol was approved by the University of Minnesota Institutional Animal Care and Use Committee. The study participants were pet dogs, and written informed consent was obtained from owners. Whole blood was collected from each dog for DNA extraction, as previously described (**Chapter 4**). Additional samples came from banked DNA at the Canine Genomics Laboratory at the University of Minnesota.

*Animals*

Four purebred Miniature Schnauzer dogs were selected for WGS: two cases and two controls. The cases were recurrent CaOx stone formers that were homozygous for the risk haplotype across the CFA37 disease-associated locus (**Chapter 4**). The controls were senior (11 and 13 yo) dogs established to be stone-free with screening abdominal radiographs to rule out radio-opaque stones and a urinalysis to rule out crystalluria. The control dogs were clear of the risk haplotype across the CFA37 locus. All four dogs were male.

A cohort of 174 Miniature Schnauzers was used for follow-up disease-association testing of variants. Seventy-nine dogs were confirmed cases and 30 were presumptive cases. Confirmed cases were diagnosed with 100% CaOx uroliths, as determined by standard stone analysis at the Minnesota Urolith Center (polarizing light microscopy and infrared spectroscopy). Presumptive cases included dogs with mixed composition stones (≥25% CaOx), as well as dogs with uroliths identified on imaging that were not analyzed but presumed to be CaOx based on radiographic appearance and urinalysis results. Sixty-

five dogs were controls. The control dogs were ≥ 9 yo with no history of CaOx urolithiasis and no evidence of urolithiasis on abdominal radiographs. One hundred and thirty-nine of the 174 dogs were SNP genotyped with the Illumina CanineHD BeadChip as described in **Chapter 4**.

A non-Schnauzer cohort of 18 dogs was used to test for the presence of variants in 4 breeds reported to have a relatively low risk for CaOx urolithiasis: the Golden retriever, Labrador retriever, Border Collie, and Rottweiler (OR < 0.2) (57, 59). The dogs in the low risk breed cohort had not been screened for stones but had no history of CaOx urolithiasis.

*WGS and variant discovery*

NextGen sequencing was performed using Illumina HiSeq 2000.[a] This provided 160 million 100 bp paired-end reads, resulting in an average coverage of 14x. The data was analyzed using the online data analysis platform Galaxy (124). The last 10 bp from the 3' end of the reads were trimmed to improve the per base sequence quality. BWA was used to map the reads to the CanFam3.1 reference sequence (125). GATK tools were used for discovery of SNPs and indels (126), and SnpEff was used to annotate the variants (127). Reads and variants were also visually inspected in the Integrative Genomics Viewer (128). For areas with inadequate coverage for sequence analysis, the CanFam3.1 reference was used to design primers to amplify the missing sequence. Standard PCR amplification was performed on a MJ Research PTC-100 thermal cycler,[b] and the products were evaluated with Sanger sequencing.

*Variant ranking and association testing*

The WGS variants present in the two cases but absent from the controls were evaluated for putative functional effect, nucleotide conservation, strength of the gene as a candidate for disease, and association with disease in the Miniature Schnauzer follow-up cohort. Gene annotation was based on the Broad Institute Improved Annotation Data v1 for the CanFam 3.1 assembly and the UCSC Genome Browser database (119, 129). The canine annotation track includes the Illumina CanineHD SNPs and SNPs from three published sources (62, 130, 131); this data was used to classify SNPs in the WGS as "known" or "novel." Nucleotide conservation was estimated using phyloP scores for basewise 100 vertebrate conservation in the "Conservation Track" of the UCSC Genome Browser (132). Gene function and pathways were obtained from the PANTHER classification system (133) and the National Center for Biotechnology Information's Gene database (134). Where indicated, MicroInspector and CENTROIDFOLD were used to determine the predicted effects of variants on miRNA binding sites and RNA secondary structure, respectively (135, 136).

For variants of interest, the CanFam3.1 reference was used to design primers to amplify a product encompassing the variant (**Table 1**). PCR amplification and sequencing was performed as described above. For one of the variants (*PLCL1* c.2703G>A), a PCR-restriction fragment length polymorphism assay was performed using 5 units of the BccI enzyme.[c]

To test for the association of variants with disease, a regression analysis was performed with CaOx stone status as the outcome and genotype and sex as predictors. Additive and recessive models were tested based on the possible modes of inheritance

determined from reviewing the GWAS SNP haplotypes (**Chapter 4**). Only genotypes for Miniature Schnauzer dogs were included in the regression analyses.

*cDNA sequencing*

Renal tissue was obtained post-mortem from a Miniature Schnauzer confirmed case that was homozygous for the CFA37 risk haplotype and a Labrador retriever that was clear of the risk haplotype. The tissue was treated with RNA*later*[d] and stored at -80° C until RNA extraction. The standard TRIzol[e] method was used for RNA extraction, and a SuperScriptII kit[f] was used to synthesize cDNA. Primers were designed to span exon-exon boundaries for *SLC39A10* such that the products could be assessed for evidence of splicing abnormalities.

## Results

The WGS provided complete coverage of the coding sequence for 14/18 genes residing in the CaOx urolithiasis-associated region of CFA37. Sanger sequencing of the missing coding regions was successful for all except exon 1 of *PLCL1*. *PLCL1* exon 1 has a high GC content, and there is missing reference sequencing flanking the 5' end (**Figure 1**).

The WGS variant discovery results are reported in **Table 2**. Coding variants in the case dog sequence were identified in three genes: *HECW2*, *GTF3C3*, and *PLCL1*. For *GTF3C3* and *PLCL1*, there was a single synonymous SNP at a base with a low phyloP conservation score. *HECW2* had a synonymous SNP that was a known variant (rs8631575) and a missense mutation that resulted in a codon change to an amino acid

that was the reference for other species including humans and mice. The *HECW2* sequence also had six occurrences of codon insertions or deletions in the case sequence for exon 8 (transcript ID: ENSCAFT00000045680). The exon containing the variants is poorly conserved in the dog relative to other species. While the gene as a whole has 93% protein identity between the canine and human reference, exon 8 only has 48% identity with multiple gaps (**Figure 2**). Not only is there poor conservation for this exon between species, but the sequencing results for additional Miniature Schnauzers and low risk breeds demonstrated a high degree of variability between dogs. All dogs had at least one codon insertion or deletion relative to the reference, and there was no consistent variant that strongly associated with disease.

Since no putative causal mutations were found in the coding sequence for any gene, non-coding sequence was examined for the top candidate gene, *SLC39A10*, to identify potential regulatory variants. The sequence for the promoter and the 5'UTR are unknown in the dog. They are believed to reside in an ~ 1 kb gap in the reference sequence (CFA37 g.5454896 – 5455927 bp, CanFam 3.1), and both long range and standard PCR across the gap was unsuccessful. However, sequence results were available for introns and the 3'UTR, and the number and type of variants are reported in **Table 3**. Multiple SNPs and indels were noted, and the two largest indels were selected for follow-up evaluation. The first was a 23 bp deletion in intron 4, and the second was a 16 bp duplication in the 3'UTR that included a SNP in both copies of the sequence (**Table 2**, ENSCAFT00000016114). To evaluate for effects on splicing, renal cDNA was amplified across exon-exon boundaries for a Miniature Schnauzer that was homozygous for the variant and a Labrador retriever. Products spanning each of the introns were successfully

obtained, and no difference was detected between the dogs. To determine if the 3'UTR variant could create other regulatory disturbances, it was evaluated for predicted effect on miRNA binding and RNA secondary structure, as described in the materials and methods. The duplication in the 3'UTR was found to cause predicted loss of cfa-miR-c452 binding and alteration in the RNA secondary structure (**Figures 3 and 4**).

The *SLC39A10* intron 4 deletion and 3'UTR duplication were selected for genotyping in a large cohort of Miniature Schnauzer dogs. The two variants were found to be in perfect linkage disequilibrium (LD) in Miniature Schnauzers, but LD was broken in one of the breeds at low risk for CaOx urolithiasis. The *SLC39A10* 3'UTR duplication was present in 3/18 of the low risk breed dogs (2 Golden retrievers and 1 Border Collie), and the intron 4 deletion was found in those 3 dogs plus 1 Labrador retriever that was clear of the 3'UTR duplication. The 3'UTR duplication was tested for strength of association with CaOx urolithiasis in 174 Miniature Schnauzers (109 cases and 65 controls). It was associated with disease in both the additive and recessive models, but the association was weak relative to other markers in the region (**Table 4**). Haplotype review for the 139 dogs with Illumina CanineHD SNP genotypes revealed that while the *SLC39A10* 3'UTR duplication was always present in the CFA37 risk haplotype, it was not exclusive to this haplotype. The same was true for the intron 4 deletion.

Though no other variants were considered likely to be causal mutations, two were selected as markers to test whether the CFA37 locus maintained disease association in the large Miniature Schnauzer cohort. These markers were located at the opposite ends of the CFA37 locus and were chosen to be near the two best candidate genes. The first marker was a 13 bp deletion ~ 0.5 Mb upstream from *SLC39A10*. The combined presence of this

upstream marker and the *SLC39A10* 3'UTR duplication accurately tagged the CFA37 risk haplotype across *SLC39A10* for 98% of the dogs with SNP genotyping. Therefore, results for these two variants were used as a proxy for the risk haplotype in the 35 dogs that were not genotyped on the CanineHD array. The second marker was the *PLCL1* synonymous SNP reported in **Table 2**. This marker uniquely tagged the CFA37 risk haplotype across *PLCL1* for all 139 dogs with SNP genotyping and was used alone to test for the haplotype in the remaining 35 dogs. Both the *SLC39A10* and *PLCL1* haplotypes were found to be significantly associated with stone risk in Miniature Schnauzers in the additive and recessive models (**Table 4**). The *PLCL1* haplotype was present in more cases and had the strongest association with disease. Clinical characteristics of the cases homozygous for the *PLCL1* haplotype were compared to those with 0-1 copies of the haplotype. The homozygous dogs were more likely to have experienced stone recurrence (OR = 3.4, p = 0.01), but they were not significantly younger at first diagnosis (7.3 yo for homozygotes versus 8.1 yo for others, p = 0.1).

## Discussion

This study reports sequencing results for 18 genes located within a region of CFA37 that has been associated with CaOx urolithiasis in Miniature Schnauzer dogs (**Chapter 4**). Based on gene function and published literature, *SLC39A10* was the top positional candidate gene. As described earlier, mutations in solute carrier genes impart risk for several other urolith types.

*SLC39A10* has not previously been evaluated for a role in urolithiasis. The encoded protein, Zip10, belongs to a family of plasma membrane metal ion transporters

and has a role in zinc (Zn) uptake (137-139). In the rat, Zip10 is expressed most abundantly in the small intestine, but it is also present in the brush border membrane of the proximal convoluted tubules (137, 139). The human gene is expressed in many tissues with little data on relative expression levels (139, 140), and tissue expression of the canine gene has not been reported.

The identification of a Zn solute carrier gene that associates with canine CaOx urolithiasis is exciting in light of a growing body of evidence that Zn plays a role in the initiation of stone formation. Zn is the most abundant heavy metal in kidney stones, and urinary Zn alterations have been reported in stone formers (141-144). Zn and calcium are similarly sized divalent cations, and calcium-containing stones have approximately 15 times higher Zn levels than non-calcium stones (144). Zn has also been shown to be present at a relatively high concentration in Randall's plaques which are mineral deposits within the renal papillae that are hypothesized to serve as a nidus for CaOx stone formation (145). Furthermore, two recent cross sectional studies revealed that high dietary Zn intake is a significant risk factor for kidney stones (146, 147).

No coding variants were found in *SLC39A10*, but two non-coding variants were assessed as putative regulatory mutations. The first was a 23 bp in an intron, and the second was a 16 bp duplication in the 3'UTR. Unfortunately, the association between these variants and stone status was relatively weak when tested in a large Miniature Schnauzer cohort ($p = 0.03 – 0.04$), and both variants were found in breeds that are reportedly at reduced risk for CaOx urolithiasis. Though these variants were considered unlikely to be causal, this does not rule out *SLC39A10* as a stone susceptibility gene. The two variants were not exclusive to the SNP risk haplotype that had been identified in the

GWAS performed in **Chapter 4**. Genotyping results for an upstream variant combined with the 3'UTR duplication were used as a marker for the risk haplotype, and this haplotype demonstrated a stronger association with stone status ($p = 0.003 – 0.005$). Though the strongest association was found for a marker in a different gene, the two markers used to define the *SLC39A10* haplotype were 0.5 Mb apart and would have failed to detect dogs that only had the haplotype across a portion of the region. The promoter and 5'UTR sequence for the gene is missing from the canine reference, and it is possible that the true mutation resides within one of these unsequenced regulatory regions. There were also numerous SNPs and small indels in the introns and 3'UTR for the gene. No evidence of aberrant splicing was found in the cDNA, but one or more of these variants could have an effect on gene expression. Quantitative RT-PCR could be used to test for differential expression of the gene between cases and controls.

One other gene in the CFA37 region was considered a good positional candidate gene for urolithiasis: *PLCL1*. There is minimal information about *PLCL1* function. It encodes a phospholipase C-like protein that lacks catalytic activity. PLCL1 binds to inositol 1,4,5-trisphosphate (IP3) and has an inhibitory role on intracellular calcium signaling (148). SNPs in *PLCL1* have been associated with hip bone size variation in women (149), and dysfunction of this gene could theoretically alter calcium release from bone. The only coding variant detected was a synonymous SNP (c.2703G>A) in exon 5 of the WGS for the case Miniature Schnauzers. This base is poorly conserved among vertebrates, and the A variant is the reference nucleotide for the rabbit and lesser hedgehog. However, the sequence for exon 1 could not be obtained with next-generation or Sanger sequencing. Exon 1 of *PLCL1* encodes the 5'UTR and the first 81 amino acids

of the protein. The *PLCL1* synonymous SNP was used as a marker to test association of this locus with CaOx urolithiasis in the Miniature Schnauzer cohort. It was found in more cases than the *SLC39A10* haplotype, and the association with stone risk was stronger ($p = 0.0001 – 0.0005$). Again, sequencing of the missing region and gene expression and/or function assays are indicated to further evaluate *PLCL1* as a susceptibility gene for stones.

The other 16 protein-coding genes in the CFA37 region did not have a functional connection to urolithiasis or calcium homeostasis, but several were uncharacterized. A coding SNP was found in *GTF3C3*, but it was synonymous and occurred at a base with a low conservation score. *HECW2*, a ubiquitin protein ligase gene, had multiple codon insertions and deletions in exon 8 of the case sequence. The canine sequence for this exon differs substantially from other vertebrates, and sequencing of additional dogs revealed that this exon has extreme variability within dogs. Based on the poor conservation of this exon within dogs and between dogs and other species, the *HECW2* variants are unlikely to be causal mutations for CaOx urolithiasis.

In conclusion, WGS failed to reveal a putative functional mutation in the genes located within the CFA37 region previously associated with CaOx urolithiasis. However, markers in this region were strongly associated with disease in a cohort of 174 Miniature Schnauzer dogs. This suggests that there is a true stone susceptibility locus on CFA37. The top candidate genes were incompletely sequenced due to problems with the reference annotation and high GC content. The causal mutation may lie within the missing region for one of these genes. Alternatively, the mutation could reside within non-coding regulatory sequence for one of the other positional genes or may be located at a more

distant location that is in LD with the markers. Further analysis of the CFA37 region is warranted.

**Footnotes**

[a]HiSeq 2000, Illumina Inc, San Diego, CA

[b]MJ Research, Inc., Watertown, MA

[c]New England BioLabs, Ipswich, MA

[d]RNA*later* Solution, Ambion Inc, Austin TX

[e]Trizol Reagent, Life Technologies, Carlsbad, CA

[f]Superscript II Reverse Transcriptase, Life Technologies, Carlsbad, CA

**Table 1.** Primers used to PCR variants of interest in the CaOx urolithiasis associated region of CFA37.

| Gene | Variant | Forward Primer | Reverse Primer | Product Size |
|---|---|---|---|---|
| *SLC39A10* | c.1491+923dup TATTCAGTTTCCTG>AT | GAAAATAGAAGAACCCAAACATGG | CAGGCCGGTAAAGCAATG | 114 bp |
| *SLC39A10* | c.571-293del TTCATATAATATGGTACTCTGTG | GTTTTTGGAACTTCCTATTTCCTG | TGCCCCCTCAATACTCAAAG | 177 bp |
| *HECW2* | Multiple variants in exon 8 (see **Table 2**) | GATTCGCTCAACGACTACCTG | ATCCTACCTGCGAGCATCCTAC | 1093 bp |
| *PLCL1* | c.2703G>A | GATAACCATTGGTGGGGATG | CAATCTCCCCATCACTTAGAGC | 423 bp |
| Region upstream to *SLC39A10* | g.8046502-8046515del GAAAGTAAAAAAA | GGATAAATGGAATCATAGAAAATGC | CCTTAGTTTTTATTTTCTGCTCATC | 103 bp |

TCAGACGCCTGCCAATCCCCACGGGCTGTCAAAACAAGTCTAGCCCCCG
CCCCCCGTCACAACAGAGCGGAGCGGAGGCCGCGTNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNGGGCAACGGAAAAAAGCGCCTTGTTTGTCCC
CCCGATAGAACCCGTTATATTGGGAAGAACGGGTTTCTGGGAAAGAGCG
CCATCATCTACCATCTCCATGCAGGGGCACAACGCTTGTAGTTTGAATCG
GTAAAGGGTGACCCAAACCGGGTCCCCGCAGAGATCAAGCGCCGGTCA
CACCGCGCCCCCGAGGACCGCCGGCCTCCCGGCCCCGAGAGATGCCGCC
TCTGCGCAAAGTTGCCGCCGCCGCCGCCGCCGCCGCCGCGGGGCGCTGA
GCGGAGTCCGGCGGGGCCGCCTGCGGGCGAGGAGCGCGGGGCGCCCGG
GGCTGGACTCCGCGGCGGGGCGTGTGCCGCCCGCCCCGGAGAGCGCGG
CCGCCGGCC**ATG**GCCCAGGGCGCGGCGGGCAGGGAGGGTCCCGCGCCG
CCCGACGCGGCCGGGGGCGAAGACGACCCCCGAGGGGGCCCGGACGCC
GCCTGCGGGGAAGGCGTGGCTGCGGCCGCCGGGGGCCGCTGGAGGGAC
CGCCGCAGCGGGGTCGCCCTGCCCGGCGCTGCGGGGGCCGCGGCTGACA
GCGAGGCGGGCCTCCTGGAGGCGGCGCGCGACCCCCCGGCGCAGCA
GCATCATCAAGGTGAGTGAGGCCGAGCCGCGGGAGCCGGGCTGCGGGG
GCCCACGGCGCGGGGACCGGCGCGGGATGCGCGGGGCCCGGGGGCATC
CATCGCCGGCTCCGCAGTGTCCCCCCCGCCCCCCGCTCTCGCCAAACCTT
CCTTCCTTCCCCGCCCGGAGGTTCCCAGACCCAGCTCTGGGCCGGGAGA
AGCAGAAGTCACTTTTTCTCCCCCGC

**Figure 1.** Exon 1 of *PLCL1* and the surrounding sequence. Exon 1 is underlined. The 5'UTR is indicated with red font and the coding sequence is in blue. N's represent unknown bases. Sequence for this exon could not be obtained likely due to a combination of the high GC content of the exon and unknown upstream sequence.

| Table 2. Sequence results for the 18 protein-coding genes residing within the CaOx urolithiasis-associated region of CFA37. | | | | |
|---|---|---|---|---|
| **Symbol** | **Name** | **Function** | **Variants detected in stone-formers*** | |
| | | | **Description** | **Predicted effect** |
| ***SLC39A10****** | Solute carrier family 39 (zinc transporter), member 10 | Metal ion transmembrane transporter activity | **ENSCAFT00000016114**<br>**1.** *3'UTR variant:* c.1491+923dup TATTCAGTTTCCTG>AT<br>**2.** *Intronic variant:* c.571-293del TTCATATAATATGGTACTCTGTG | **1.** Putative disruption of a miRNA binding site and alteration of the mRNA secondary structure<br>**2.** Unknown. Low to moderate conservation of bases. |
| *DNAH7* | Dynein, axonemal, heavy chain 7 | Microtubule motor activity | No coding variants | |
| *STK17B* | Serine/threonine kinase, 17b | Protein kinase activity | No coding variants | |
| *HECW2* | HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2 | Ubiquitin-protein ligase activity | **ENSCAFT00000045680**<br>**1.** c.93C>T<br>**2.** c.793T>A, p.265S>T<br>**3.** c.1334_1335 insAGCAGGAGCAGGACCAGG, p.444_445insSRSRTR<br>**4.** c.1492_1493insGCG, p.98_99insA<br>**5.** c.1542_1543insCAG, p.514_515insQ<br>**6.** c.1680_1681insGGGGCC, p.560_561insGA<br>**7.** c.1773_1774delGAGCAG, p.596_597delEQ<br>**8.** c.1824_1825delGAGCCC, | **1.** None. Synonymous SNP and known variant (rs8631575).<br>**2.** None. Threonine is the reference amino acid at this codon in several species including humans and mice.<br>**3-8.** Unknown. This exon has poor conservation in the dog relative to other vertebrates. Multiple |

| | | | | |
|---|---|---|---|---|
| | | | p.608_609delPE | codon insertions and deletions are also present in the controls. |
| *CCDC150* | Coiled-coil domain containing 15 | Uncharacterized | No coding variants | |
| *GTF3C3* | General transcription factor IIIC, polypeptide 3, 102kDa | Sequence-specific DNA binding transcription factor activity | **ENSCAFT00000043408**<br>**1.** c.339G>A | **1.** None. Synonymous SNP and low conservation base. |
| *C2orf66* | Chromosome 2 open reading frame 66 | Uncharacterized | No coding variants | |
| *PGAP1* | Post-GPI attachment to proteins 1 | Intracellular protein transport; proteolysis | No coding variants | |
| *ANKRD44* | Ankyrin repeat domain 44 | Protein binding | No coding variants | |
| *SF3B1* | Splicing factor 3b, subunit 1, 155kDa | mRNA splicing | No coding variants | |
| *COQ10B* | Coenzyme Q10 homolog B (S. cerevisiae) | Required for function of coenzyme Q | No coding variants | |
| *HSPD1* | Heat shock 60kDa protein 1 (chaperonin) | Protein folding | No coding variants | |
| *HSPE1* | Heat shock 10kDa protein 1 | Protein folding | No coding variants | |
| *MOB4* | MOB family member 4, phocein | Kinase activity | No coding variants | |
| *RFTN2* | Raftlin family member 2 | Uncharacterized | No coding variants | |

| | | | | |
|---|---|---|---|---|
| *MARS2* | Methionyl-tRNA synthetase 2, mitochondrial | Aminoacyl-tRNA ligase activity | No coding variants | |
| *BOLL* | Boule-like RNA-binding protein | RNA binding; gamete generation | No coding variants | |
| ***PLCL1*** | Phospholipase C-like 1 | Calcium ion binding; small GTPase regulator activity | **ENSCAFT00000017444**<br>**1.** c.2703G>A | **1.** None. Synonymous SNP and low conservation base. |

Genes are listed in the order in which they are annotated from CFA37 g.7061552 – 10653612 bp CanFam 2 and g.4061607 – 7653685 bp CanFam 3.1. The top two candidate genes are bolded and highlighted in blue font.

*The reported variants were present in the two cases and absent from the controls in the WGS. All variants were present in a homozygous state. Coding variants only were assessed for all except the top candidate gene, *SLC39A10*. Variants in the UTRs and introns are also reported for this gene.

**Coverage over exon 1 of *SLC39A10* (5'UTR) and *PLCL1* (5'UTR and coding) could not be obtained.

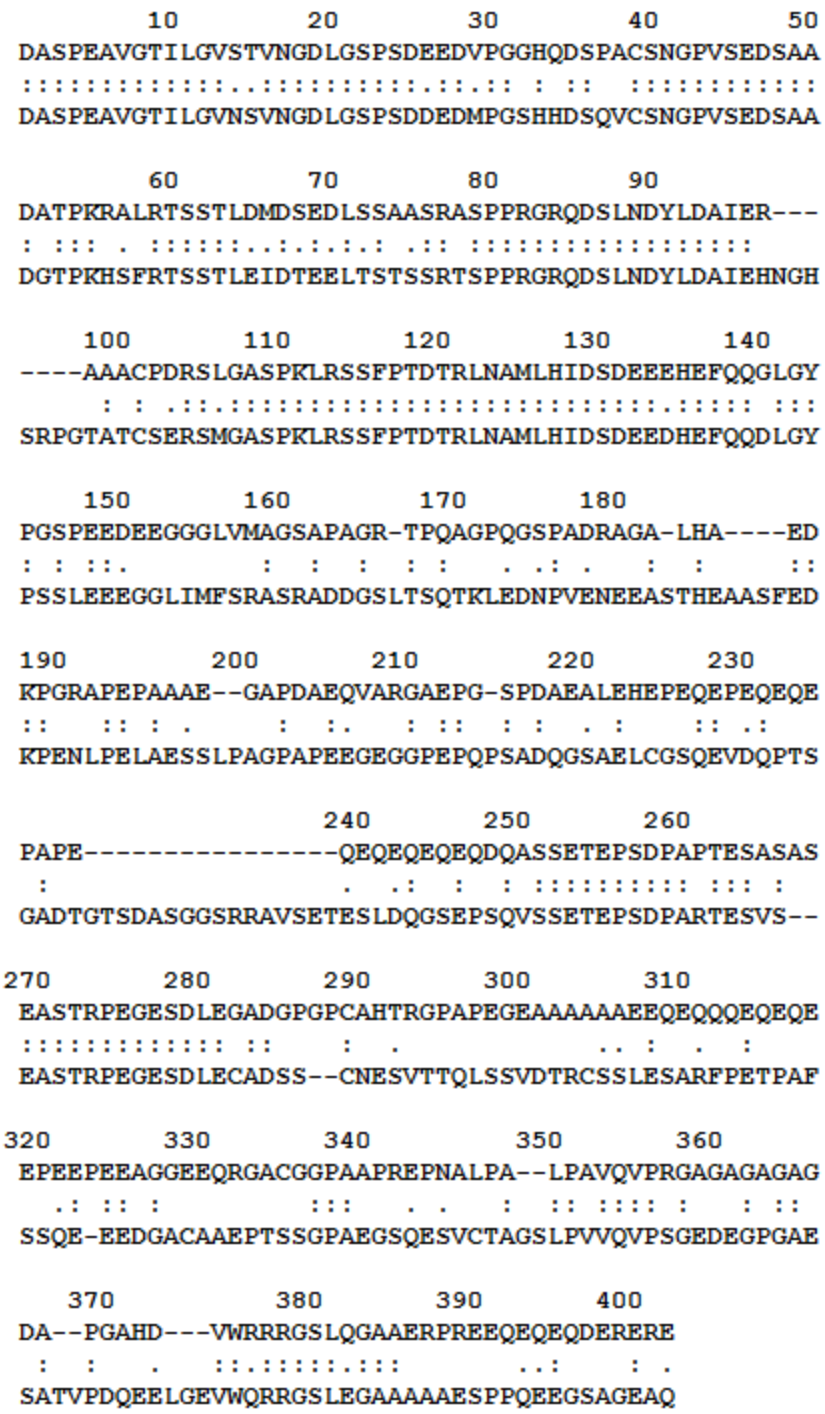


**Figure 2.** Exon 8 of *HECW2* has low protein sequence similarity between dogs and other species. Alignment between the canine and human reference sequences is displayed with canine as the upper sequence and human as the lower.

**Table 3.** Non-coding SNPs and indels in *SLC39A10*. These variants were present in two Miniature Schnauzer dogs with a history of CaOx urolithiasis and were absent from two stone-free control Miniature Schnauzers. See Table 2 for a detailed description of the two largest indels. The UCSC Broad Institute CanFam 3 Improved Annotation Data v1 was used to determine if SNPs were known or novel.

| Variant Type | Intron | 3'UTR |
|---|---|---|
| **SNP** | | |
| **Known** | 15 | 4 |
| **Novel** | 20 | 6 |
| **Indel** | | |
| **<5 bp** | 11 | 5 |
| **5-10 bp** | 1 | 0 |
| **>10 bp** | 1 | 1 |

***cfa-miR-c452:*** AGUCAAA:GGAGACGUUUGUCAA
***Control:*** TATTCAGTTTTCCTGTATTAACAGTACTAAACT

***cfa-miR-c452:*** AGUCAAA:GGAGA::::::::::::::::::::::::::::::::::::CGUUUGUCAA
***Case:*** TATTCAGTTTTCCTATTATTCAGTTTTCCTATATTAACAGTACTAACT

**Figure 3.** A 16 bp duplication in the 3'UTR of *SLC39A10* was identified in the WGS of two Miniature Schnauzer dogs with CaOx urolithiasis and was absent from the two stone-free controls. The duplication is in red font, and both copies of the duplicated sequence contain a SNP (underlined) in the case dogs. This variant is predicted to cause loss of a microRNA (blue) binding site.

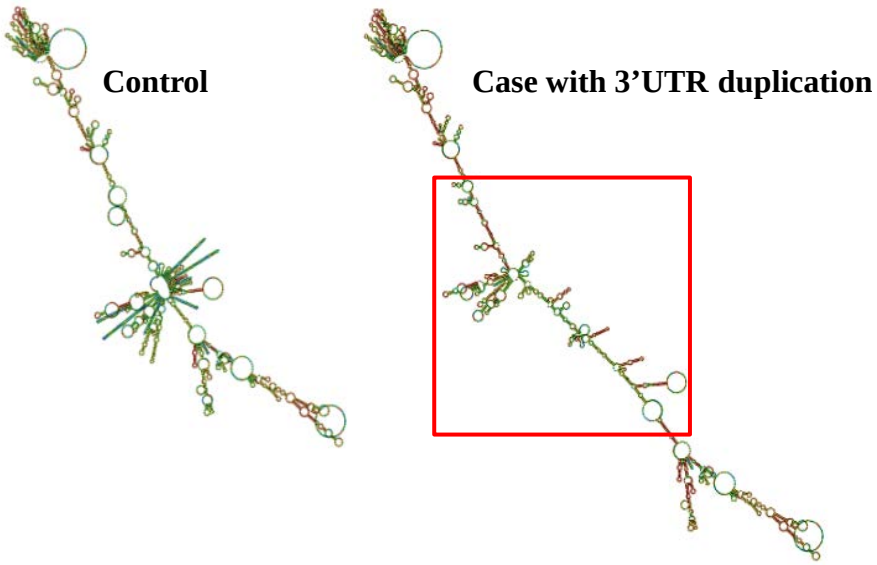


**Figure 4.** A 16 bp duplication in the 3'UTR of *SLC39A10* causes predicted alterations in mRNA secondary structure (red box) relative to the control/reference

**Table 4.** Association of a deletion upstream to *SLC39A10*, a 16 bp duplication in the 3'UTR of *SLC39A10* and a synonymous SNP in *PLCL1* with CaOx urolithiasis in 174 Miniature Schnauzers: 109 cases and 65 controls. The number of dogs with the variants (proportion) is reported, and the p-value is for the results of a regression with genotype and sex as predictors of stone status. Both additive and recessive models were tested. WT denotes the "wild type" reference sequence, and V denotes the variant genotype.

| | ***SLC39A10*** **3'UTR duplication** | | ***SLC39A10*** **Risk haplotype** | | ***PLCL1*** **Synonymous SNP** | |
|---|---|---|---|---|---|---|
| **Genotypes** | **Cases** | **Controls** | **Cases** | **Controls** | **Cases** | **Controls** |
| **WT/WT** | 35 (0.32) | 28 (0.43) | 50 (0.46) | 41 (0.63) | 41 (0.38) | 35 (0.54) |
| **WT/V** | 45 (0.41) | 28 (0.43) | 41 (0.38) | 22 (0.34) | 44 (0.40) | 29 (0.45) |
| **V/V** | 29 (0.27) | 9 (0.14) | 18 (0.16) | 2 (0.03) | 23 (0.21) | 1 (0.01) |
| **p-value** | | | | | | |
| **Additive** | **0.03** | | **0.003** | | **0.0005** | |
| **Recessive** | **0.04** | | **0.005** | | **0.0001** | |

# CHAPTER 6

## Urinary trace metals in canine calcium oxalate urolithiasis

**Urinary trace metals in canine calcium oxalate urolithiasis**

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## Summary

Data from human, laboratory animal, and *in vitro* studies have demonstrated an effect of multiple trace metals in the crystallization process, and epidemiologic studies in humans have found relationships between urinary concentrations of metals such as zinc (Zn) and cadmium (Cd) and CaOx stone risk. Furthermore, a recent genome-wide association study in dogs identified a susceptibility locus for CaOx urolithiasis that contains a metal ion solute carrier gene, *SLC39A10*. The aim of this study was to test for a relationship between urinary trace metals, stone status, and *SLC39A10* genotype in dogs. We hypothesized that urinary levels of Zn and Cd would be higher in stone formers and that *SLC39A10* genotype would affect these levels.

No significant relationship was present between urinary Zn or Cd and either stone status or *SLC39A10* genotype. However, urinary iron (Fe) and vanadium (V) were higher in stone-formers than controls. These results were not driven by a particular breed, diet, or historical illness. Neither urinary Fe nor V levels had a relationship to the *SLC39A10* genotype, but the *SLC39A10* genotype did predict urinary calcium and strontium (Sr) levels. Urinary calcium and Sr were moderately correlated, and these elements have been previously reported to colocalize in stones. It is unknown whether Sr is simply a marker of calcium homeostasis or could play a direct role in stone formation. The results of this study suggest that Fe and V should be investigated as potential environmental risk factors for CaOx urolithiasis and that *SLC39A10* could play a role in lithogenesis by altering the handling of calcium and Sr.

## Introduction

Calcium oxalate (CaOx) urolithiasis is a common, painful, and difficult to treat urinary tract disease in dogs. Medical dissolution of CaOx stones is not possible; removal is required for cure. Preventative options for this disease have low efficacy, and recurrence rates are high (107). The incidence of CaOx urolithiasis is on the rise both in dogs and people, and it is hypothesized that shared environmental risk factors are responsible (8, 58, 150). Novel research approaches are needed to elucidate environmental risk factors for CaOx urolithiasis.

Several metals have been implicated in lithogenesis, but the relationships between individual metals and stone risk are not well understood. Zinc (Zn), strontium (Sr), and iron (Fe) are found in appreciable amounts in kidney stones, and these metals are detected at greater quantities in calcium-containing stones than other stone types (144). Zn is the most abundant of the three, and it is found at a relatively high concentration in Randall's plaques (mineral deposits within the renal papillae that are associated with CaOx stones) (145). Recent studies have suggested a link between high dietary Zn and CaOx stones (146, 147). However, the reports of urinary Zn alterations in human stone formers show conflicting results. Some studies report higher urinary Zn in patients with kidney stones, while others report lower (141-143). Furthermore, *in vitro* studies have reported that Zn has an inhibitory effect on CaOx crystallization (151).

Metals that are not commonly detected within uroliths can still play an important role in the disease. Cadmium (Cd) is a classic example. It is an environmental toxin that causes renal damage and alters calcium homeostasis. Low level Cd exposure in people is

associated with risk for osteoporosis, hypercalciuria and nephrolithiasis, without other overt signs of toxicity (152, 153).

To the authors' knowledge there are no published reports on the relationship between urinary trace metals and CaOx urolithiasis or hypercalciuria in dogs. The need for such investigations was recently highlighted by our canine genetic studies. We performed a genome-wide association study (GWAS) and identified a susceptibility locus for canine CaOx urolithiasis that harbors a renally- and intestinally-expressed metal ion solute carrier gene, *SLC39A10* (**Chapters 4 and 5**). The encoded protein, Zip10, is a known transporter of Zn and Cd (139). Thus, not only could these metals be general environmental risk factors for stones, but genetic variation in metal uptake and elimination could contribute to risk.

There were two objectives of this study. The first was to evaluate the relationship between urinary trace metals, urinary calcium, and CaOx stone status in a large population of dogs from diverse breed groups. The second was to test for a relationship to between a CaOx stone risk haplotype across *SLC39A10* and urinary trace metals. We hypothesized that urinary Zn and Cd would be higher in stone-formers and would be associated with *SLC39A10* genotype.

## Materials and Methods

### *Study Population*

The study took place at the Veterinary Medical Center, University of Minnesota (VMC UMN). Dogs were recruited from February 2011 to March 2014. Cases were defined as dogs with a history of uroliths composed of CaOx (≥70% of the central core),

as determined by standard stone analysis at the Minnesota Urolith Center (polarizing light microscopy and infrared spectroscopy). Controls were ≥ 8 yo and had no history of CaOx uroliths. Controls were screened with abdominal radiographs to rule out radio-opaque uroliths and a urinalysis to rule out CaOx crystalluria.

For both groups, dogs were excluded if they had received glucocorticoids within the past week or another drug with known effects on urinary calcium excretion (i.e. furosemide, thiazide diuretics, levothyroxine, theophylline, potassium citrate) within the past 24 hours. Dogs were also excluded if they had a clinical diagnosis of a disease that alters urinary calcium excretion (i.e. hyperparathyroidism, hypercalcemia of malignancy, hyperadrenocorticism, diabetes mellitus, osteolytic disease, granulomatous disease). The current diet was recorded for each dog and classified into one of three groups: Hill's Prescription Diet canine u/d,[a] Royal Canin Veterinary Diet canine urinary SO,[b] or other. Written informed consent was obtained from the dog owners, and the study protocol was approved by the University of Minnesota Institutional Animal Care and Use Committee.

### *Urinary Measurements*

Owners were instructed to withhold food, but not water, for 12-18 hours prior to sample collection. All dogs had voided at least once prior to sample collection to insure that the research urine sample was never the first micturition of the day. Urine was collected by free catch, cystocentesis, or catheterization. Unfiltered 1-3 ml urine aliquots were immediately analyzed for calcium (spectroscopy and the calcium sensitive dye Arsenazo II) and creatinine (modified Jaffe procedure) (**Chapter 3**). A 4 ml urine aliquot was filtered through a 0.22 µm polyethersulfone membrane,[c] stored at -80° C, and

analyzed for trace metals within 18 months. Urinary metal analysis was performed using inductively coupled plasma mass spectrometry (154). Twelve metals were included in the analysis: barium (Ba), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), rubidium (Rb), strontium (Sr), vanadium (V), and zinc (Zn). Spot urinary element-to-creatinine ratios (e.g. UCa/Cre, Zn/Cre, Cd/Cre) were calculated for each dog and are reported in mg/g for UCa/Cre and µg/g for the metals.

### *SLC39A10 Genotyping*

SNP genotyping results from the Illumina CanineHD array were used to evaluate dogs for the risk haplotype across *SLC39A10* identified and described in **Chapters 4 and 5**. For dogs not on the array, PCR results for a 13 bp deletion upstream to *SLC39A10* and a 16 bp duplication in the 3'UTR of the gene were used in combination as a marker for the risk haplotype (see **Chapter 5**). Only Miniature Schnauzer dogs were genotyped for the *SLC39A10* haplotype.

### *Statistical Analysis*

Ages are reported as mean ± standard deviation. The results of the urinary element-to-creatinine ratios were visually inspected with Q-Q plots. The majority of the measurements did not appear follow a normal distribution. Therefore, Wilcoxon rank sum tests were used to compare measurements between case and control groups with the Bonferroni-Holm correction for multiple comparisons. Each variable with a corrected p-value $\leq 0.25$ was analyzed further with multiple regression. Prior to the regression analyses, the data was transformed with the natural logarithm function. Zero values were

present for some of the urinary metal measurements. To manage this, the minimum non-zero value was added to each dog's measurement for that metal such that the entire group was shifted up by the minimum prior to log-transformation. Regression analyses were performed for the log-transformed urinary element-to-creatinine ratios with stone status (case versus control), breed, diet (Hill's u/d, Royal Canin SO, and other), age at the time of urine sampling (in years), and sex (male versus female) as predictors. The urinary element-to-creatinine ratios with a significant relationship to urolithiasis were also analyzed together in a model as predictors of stone status. Sex was included as an additional predictor in the stone model. Variance inflation factors (VIF) were calculated to assess for multicollinearity, and Pearson's correlation coefficients (r) were determined for the urinary variables used as predictors of stone status. For each of the multiple regressions, stepwise selection of predictors was performed in both directions, and an Akaike Information Criterion (AIC) score was calculated for each model. The model with the lowest AIC was selected as the best fit. The significance of each predictor in the linear models was assessed using Type II tests (ANCOVA).

The relationships between the *SLC39A10* risk haplotype and urinary element-to-creatinine ratios were tested using linear models. The urinary data was log-transformed as described above. Simple regressions were performed with genotype (0, 1, or 2 copies of the risk haplotype) as the predictor and urinary element-to-creatinine as the outcome. Based on these results, urinary variables were selected for multiple regression analysis with genotype and the aforementioned predictors (stone status, sex, age at the time of urine sampling, and diet). *SLC39A10* genotype and sex were also tested as predictors of

stone status. Selection of predictors and testing of model fit were performed as described above. Pearson's r was calculated for urinary variables of interest.

All analyses were performed using the R software for statistical computing,[d] and p-value of <0.05 was considered significant.

## Results

*Study participant characteristics*

One hundred and six dogs (64 cases and 42 controls) were enrolled in the study. The most common breeds were the Miniature Schnauzer (37), Bichon Frise (22), Shih Tzu (14), mixed breed (10), Lhasa Apso (5), Yorkshire terrier (3), and Rat terrier (2). See **Table 1** for additional breeds and details on the clinical characteristics of the study participants. The majority of the dogs in both groups were males (81% of cases and 55% of controls). Twenty-eight cases (44%) had cystoliths at the time of sample collection and had their stones removed later that same day. Twenty of the cases (31%) had suffered from recurrent CaOx uroliths.

*Urinary metals and stone status*

The median element-to-creatinine values for the case and control groups and p-values for the Wilcoxon rank sum test are reported in **Table 2**. Three urinary variables had a Bonferroni-Holm corrected p-value $\leq 0.25$ and were selected for regression analysis: UCa/Cre ($p < 0.001$), UFe/Cre ($p = 0.13$), UV/Cre ($p = 0.07$). For all three, the cases had higher median values than the controls (**Table 2, Figure 1**). The results for the full and reduced models for each of the urinary outcomes are reported in **Table 3**. For

UCa/Cre and UV/Cre, the best models had stone status as the sole predictor ($p < 0.001$ and 0.004, respectively). The best model for UFe/Cre included stone status and diet. While there was a positive relationship between UFe/Cr and CaOx urolithiasis ($p = 0.0003$), there was a negative correlation between UFe/Cr and the Hill's u/d diet ($p = 0.005$).

UCa/Cre, UFe/Cre, UV/Cr, and sex were placed into a regression analysis as predictors of CaOx urolithiasis. None of these variables were highly correlated ($r < 0.11$), and they had low VIFs in the model (<1.05). The CaOx urolithiasis regression is presented in **Table 4**. The full model had a better fit than any of the reduced models, and all of the predictors in the model had a significant relationship with stone status. Sex had the greatest estimate for effect on stone risk, followed by UCa/Cre, UFe/Cre, and lastly, UV/Cre.

The medical history for the dogs with the 10 highest values for UFe/Cre and UV/Cre were examined to determine if there was any overt shared characteristic or exposure history (diet or medication). The 10 highest measurements came from dogs of 6 different breeds for UFe/Cre and 8 for UV/Cre, and the dogs were consuming 9 and 7 different diets, respectively. One of the dogs with high UFe/Cre was receiving doxycycline for a cough, but none of the other dogs were on any medications or supplements. There were no apparent shared characteristics apart from stone status (9/10 were cases for both groups).

Though UCd/Cre did not differ between case and control groups, the data was inspected for dogs with UCd/Cre in ranges associated with increased risk for renal damage (≥ 1 μg/g) (155), urolithiasis (> 1 μg/g) (153), or low bone mineral density (> 3

µg/g) (156). A single case had a high UCd/Cre at 3 µg/g. This dog's level was > 30 times the median for the other dogs (0.087, range = 0 – 0.69 µg/g). The dog was a male neutered terrier mix with a history of CaOx stones at 3 and 8 yo. He was adopted by a new owner at 8 yo, and the study urine sample was obtained at 9 yo. The dog was eating a therapeutic liver diet[e] at the time of the urine sample, but he was healthy aside from his stone history and was not on any medications. A household dog was also a recurrent CaOx stone-former. The household dog had the same dietary history as the high UCd/Cre dog for the year prior to study participation. However, the household dog had low urinary Cd (UCd/Cre = 0.076).

*Urinary metals and SLC39A10 genotype*

**Table 5** includes the results of simple regression analyses using genotype for the *SLC39A10* risk haplotype to predict urinary variables and stone status in 37 Miniature Schnauzers. Eighteen dogs were clear of the haplotype, 15 were carriers (heterozygous), and 4 dogs were homozygous for the risk haplotype. No urinary variable reached significance after correction for multiple testing, but two were selected for further evaluation with multiple regression analysis: UCa/Cre ($p = 0.15$) and USr/Cre ($p = 0.30$). Both variables had a positive relationship with the *SLC39A10* risk haplotype (**Figure 2**), and they were moderately correlated with each other ($r = 0.62$, $p < 0.001$, **Figure 3**). The results for the full and reduced models for UCa/Cre and USr/Cre are reported in **Table 6**. The best model for UCa/Cre for included genotype ($p = 0.07$) and stone status ($p = 0.05$) as predictors, whereas the USr/Cre reduced model only included genotype ($p = 0.03$). *SLC39A10* genotype was also found to predict stone status (**Table 6**, $p = 0.04$).

## Discussion

The present study demonstrates an association between urinary calcium, Fe, and V levels and canine CaOx urolithiasis. Hypercalciuria has been previously documented as an important metabolic abnormality in stone formers (**see Chapter 3**), and UCa/Cre was the strongest urinary predictor of stone status in this study. However, to the authors' knowledge, this is the first published report investigating urinary trace metals in canine CaOx urolithiasis. Both urinary Fe and V had a positive relationship with stone status that was independent of urinary calcium. Ten other metals did not differ between stone-formers and stone-free controls. These findings were unexpected. We had initiated the study with the hypothesis that urinary Zn and Cd would be higher in stone formers, and we had not predicted alterations in Fe or V. Nevertheless, the results document exciting novel associations that could shed light on the pathogenesis of canine CaOx urolithiasis.

Fe is an essential trace element. It is the third most abundant metal in human kidney stones, after Zn and Sr, and is more abundant in calcium containing stones than other stone types (144). Ferric ions can inhibit CaOx crystal development *in vitro*, but this effect does not occur when physiological levels of citrate are present (157). Rather than having a direct effect on lithogenesis, Fe could play a role in promoting CaOx stone risk by increasing bone resorption. Rats with dietary Fe overload have increased markers of bone turnover and twice the urinary calcium levels of age and sex-matched controls (158). While this is intriguing, the correlation between urinary Fe and calcium for the dogs in this study was poor ($r = 0.08$, $p = 0.4$). An interesting finding was that one of the common therapeutic stone-prevention diets[a] had a negative correlation with urinary Fe. This diet was only prescribed after stone diagnosis, and it is unknown if the cases had a

higher urinary Fe prior to the diet change. Thus, the association between urinary Fe and stone risk could be greater than estimated by this data. A more in-depth discussion of this limitation is included later.

For some mammals, V is an essential nutrient, but it has not reached this status in humans (159). It is inconsistently detected in human kidney stones (144, 160, 161). A positive correlation has been noted between urinary and stone V content for calcium phosphate and magnesium phosphate stones, but this relationship is not present for CaOx stones (161). Data on a direct role of V in lithogenesis is lacking (151), but V could be a biomarker of altered bone turnover or have an indirect effect on stone risk. The highest tissue levels of V are found in bone, where V associates with phosphate, upregulates alkaline phosphatase activity, increases mineralization, stimulates osteoblast differentiation, and may act as a growth-factor mimetic (162, 163). At high doses, oral V supplementation raises serum calcium in rats (164). We did not test for an association between urinary V and blood calcium in this study, but there was no significant correlation with urinary calcium ($r = 0.11$, $p = 0.3$).

Another theoretical link between V and urolithiasis comes from the renal effects of V. The kidney is the second major site of V concentration, and multiple *in vitro* and *in vivo* studies have shown it to inhibit the renal Na/K-ATPase which is important for Na and water resorption (159, 165). Disturbances in salt or water intake and excretion can impact stone risk (166). The V-induced inhibition of renal Na/K-ATPase decreases urinary water resorption, and the resultant drop in urine concentration should reduce crystal aggregation. However, there may be other consequences of the renal V effects that promote stone formation.

The urinary levels of the two metals that we had specifically set out to test, Zn and Cd, did not differ between CaOx cases and stone-free control dogs. Reports on urinary Zn alterations in human nephrolithiasis have been conflicting (141-143), but two studies have found higher dietary Zn intake in patients with stones relative to controls (146, 147). Zn is primarily eliminated through the intestinal tract and changes in dietary Zn intake have relatively minor effects on urinary Zn compared with fecal Zn (167). Therefore, urinary Zn is not the most sensitive method to detect alterations in Zn balance and cannot be used to rule out a role of Zn in urolithiasis.

Unlike the other metals analyzed, Cd is a toxin with a well-documented effect on urolithiasis risk. It accumulates in renal tubular cells and causes hypercalciuria through inhibition of calcium resorption, effects on calcium uptake by bone, and alterations in vitamin D metabolism (152, 168). Low level exposure is associated with increased stone risk without other classic signs of toxicity (153). Though urinary Cd was not associated with CaOx urolithiasis in this dog population, one case dog had a UCd/Cre that was three times the level associated with stone risk in humans. A household dog that was also affected by CaOx stones had normal urinary Cd despite eating the same diet as the high Cd dog for the past year. However, the high Cd dog's dietary and environmental history prior to age 8 was largely unknown. Renal tissue accumulates Cd, and it is very slowly eliminated through the urine. Urinary Cd is therefore considered a biomarker of lifetime Cd exposure (169). Toxicokinetic studies of chronic oral Cd dosing in dogs has estimated the biological half-life at 1-2 years for a two compartment model, and human data suggests that it may take 10-30 years for elimination from the renal cortex and other tissues (170, 171). Thus, the dog with high Cd may have had an early exposure to Cd that

contributed to development of CaOx uroliths. Since only this one dog out of 106 study participants had a UCd/Cre in a range associated with pathology in people, it can be concluded that Cd toxicity is uncommon for dogs in the region where the study was conducted. Further studies involving greater numbers of dogs from diverse regions of the country are needed to determine whether Cd is a substantial environmental risk factor for canine CaOx urolithiasis.

One of the motivating factors for this study was the identification of a metal ion transporter gene, *SLC39A10*, at a susceptibility locus for CaOx urolithiasis. The encoded Zip10 protein is best characterized as a Zn transporter but likely has affinity for a number of metal ions. There was no evidence for a relationship between the risk haplotype across *SLC39A10* and UZn/Cre in this study. Zip10 is expressed in a number of tissues including the intestinal tract. As discussed earlier, the intestinal tract is the primary site of Zn uptake and elimination, and urinary Zn levels may not have sufficient sensitivity for detecting disorders in Zn homeostasis.

While urinary Zn did not show a significant relationship to *SLC39A10* genotype, higher urinary calcium and Sr levels were found in dogs with the risk haplotype. Stone status had a stronger relationship to UCa/Cre than genotype, but the association of these two predictors makes interpretation difficult. Genotype was the only significant predictor of USr/Cre in a model that included stone status, sex, age, and diet. Strontium is a non-essential trace element. It is the second most abundant metal detected in kidney stones and Randall's plaques (144, 145, 172). Strontium has a similar size and charge to calcium. It co-localizes with calcium in kidney stones (172) and has been theorized to serve as a nidus for stone formation (173). The absorption and excretion of Sr follow that

of calcium (174). In fact, oral Sr loading is used to study calcium metabolism in patients with idiopathic hypercalciuria (175, 176). In the present study, USr/Cre and UCa/Cre had a moderate positive correlation ($r = 0.62$, $p < 0.001$). The higher urinary Sr and calcium levels in dogs with the risk haplotype could be a marker of *SLC39A10* induced intestinal hyperabsorption or decreased renal resorption of these elements. Strontium is present at trace levels in bone, and bone resorption could be another mechanism behind the higher urinary levels. However, Sr has a mild suppressive effect on bone resorption and would not be predicted to be the cause of increased bone turnover (177). A direct role of Sr in lithogenesis is also unlikely, given the absence of data to support this effect.

There are a couple of important limitations of this study. Urine samples were obtained from nearly half of the cases at the time that a stone was present, but some dogs had been diagnosed with stones more than a year prior to study enrollment. For these dogs, a metal exposure may have been missed if the metal was eliminated from the body before the urine sample was obtained. This is most concerning for elements that are tightly regulated by the body and have short elimination half-lives, such as Ca, Zn, and Fe. The time gap between stone formation and the urine sample also complicates the ability to determine the source of the metal exposure and when the exposure occurred relative to stone formation.

In conclusion, urinary Fe and V were found to have a positive relationship with CaOx urolithiasis. The mechanism behind the association is unclear but could theoretically be related to abnormalities in bone turnover. Serum and stone metal quantification along with a more in-depth nutritional analysis may help better elucidate the relationship between these metals and stones. A prospective study could also be

designed to determine relative risk of forming stones in dogs with high versus low urinary Fe and V levels. No association was found between urinary Zn and stone risk in this study, and high urinary Cd was rare. Genotype across the *SLC39A10* metal ion transporter gene had a significant relationship to urinary calcium and Sr in Miniature Schnauzer dogs. Further studies are needed to identify a functional mutation and prove *SLC39A10* as a susceptibility gene for CaOx urolithiasis. Knowledge of the role of trace metals in CaOx stone formation could have implications for the formulation of preventative and therapeutic veterinary diets.

**Footnotes**

[a]Prescription Diet canine u/d, Hill's Pet Nutrition Inc, Topeka, KS
[b]Royal Canin Veterinary Diet canine urinary SO, Waltham Centre for Pet Nutrition, Leicestershire, UK
[c] MJ Research, Inc., Watertown, MA
[d]R Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/
[e]Prescription Diet canine l/d, Hill's Pet Nutrition Inc, Topeka, KS

**Table 1.** Characteristics of the 106 study dogs. Cases are dogs with confirmed CaOx urolithiasis, and controls are stone-free as determined by abdominal radiography. Age at the time of urine sampling is reported as mean ± standard deviation.

| Stone Status | Breed | Sex | Age | Diet |
|---|---|---|---|---|
| **Cases (64 dogs)** | 17 Miniature Schnauzers<br>10 Bichons Frise<br>7 Shih Tzus<br>10 Mixed breed dogs<br>5 Lhasa Apsos<br>3 Yorkshire terriers<br>2 Rat terriers<br>1 each: Chihuahua, Cocker Spaniel, Fox terrier, Havanese, Jack Russell terrier, Maltese, Pomeranian, Pug, Shetland Sheepdog, Welsh terrier | 52 M, 12 F | 8.9 ± 2.5 yo | 15 Hill's u/d<br>10 Royal Canin SO<br>40 Other |
| **Controls (42 dogs)** | 20 Miniature Schnauzers<br>12 Bichons Frise<br>7 Shih Tzus<br>1 each: Mixed breed, Standard Schnauzer, Miniature Poodle | 23 M, 19 F | 10.3 ± 1.8 yo | 1 Royal Canin SO<br>40 Other |

**Table 2.** Urinary calcium and trace metal-to-creatinine ratios in dogs with CaOx urolithiasis (cases) and stone-free controls. Values are reported in mg/g for UCa/Cre and µg/g for the urinary metal-to-creatinine ratios.

| Variable | Control Median | Case Median | Raw p-value | Bonferroni-Holm p-value |
|---|---|---|---|---|
| **UCa/Cre** | **31** | **70** | **1.2E-06** | **1.5E-05** |
| **UZn/Cre** | 233 | 210 | 0.60 | 1 |
| **USr/Cre** | 88 | 115 | 0.05 | 0.39 |
| **UCd/Cre** | 0.084 | 0.089 | 0.9 | 1 |
| **UV/Cre** | **2.9** | **6.8** | **0.006** | **0.07** |
| **UCr/Cre** | 0.67 | 0.71 | 0.28 | 1 |
| **UFe/Cre** | **14** | **19** | **0.01** | **0.13** |
| **UMn/Cre** | 0.18 | 0.31 | 0.18 | 1 |
| **UCo/Cre** | 2.6 | 1.5 | 0.04 | 0.36 |
| **UNi/Cre** | 8.0 | 8.3 | 0.51 | 1 |
| **UCu/Cre** | 115 | 144 | 0.05 | 0.39 |
| **URb/Cre** | 2106 | 1723 | 0.03 | 0.32 |
| **UBa/Cre** | 8.0 | 8.1 | 0.83 | 1 |

**Figure 1.** Box and whisker plots of fasting spot urinary **A**) calcium-to-creatinine (UCa/Cre, mg/g), **B**) iron-to-creatinine (UFe/Cre, µg/g), and **C**) vanadium-to-creatinine (UV/Cre, µg/g) ratios for dogs with a history of CaOx stones (cases) and breed-matched stone-free dogs (controls). The boxes represent the interquartile range ($25^{th}$ – $75^{th}$ percentile), the horizontal line within the boxes represents the median, and the whisker bars extend to 1.5 times the interquartile range. Circles represent outliers. Bonferroni-Holm corrected p-values are reported.

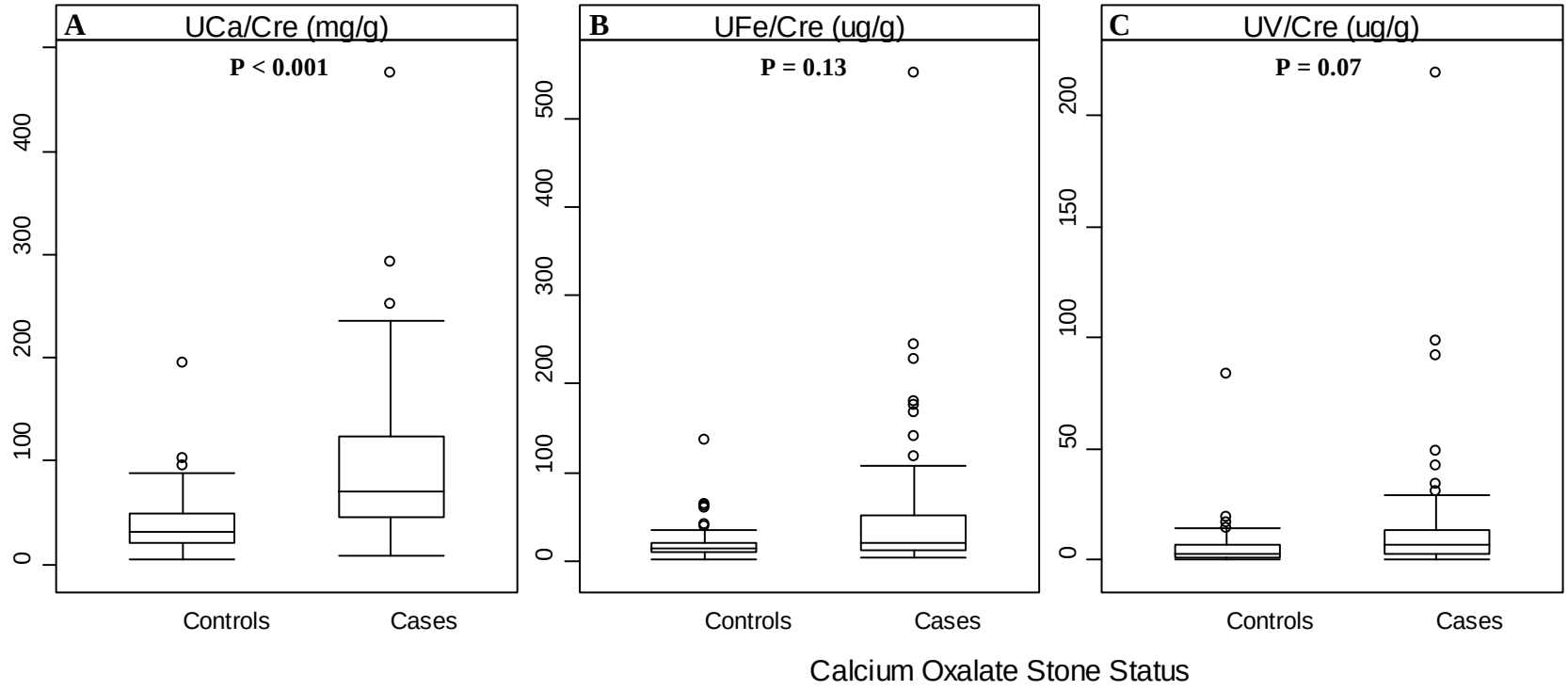

**Table 3.** Regression and analysis of covariance (ANCOVA) results for the effects of CaOx urolithiasis and environmental factors on log-transformed urinary calcium-to-creatinine (UCa/Cre), iron-to-creatinine (UFe/Cre), and vanadium-to-creatinine (UV/Cre) ratios. The reduced model represents the model with the best AIC as determined by stepwise selection of predictors. For variables with 1 degree of freedom, the status corresponding to the estimate is in parentheses. Estimates for breed effects are not reported due to the large number of categories.

| | Estimate | Standard Error | T value | Degrees of Freedom | P-value |
|---|---|---|---|---|---|
| **logUCa/Cre** | | | | | |
| **Full Model, AIC = 56** | | | | 6 | **0.003** |
| Stone status (case) | 1.1 | 0.26 | 4.3 | 1 | **9E-05** |
| Sex (male) | -0.39 | 0.25 | -1.5 | 1 | 0.2 |
| Breed | NA | NA | NA | 18 | 1 |
| Age | 0.02 | 0.06 | 0.34 | 1 | 0.7 |
| Diet | | | | 2 | 0.5 |
| Hill's u/d | 0.50 | 0.42 | 1.2 | | 0.1 |
| Royal Canin SO | 0.14 | 0.46 | 0.3 | | 0.5 |
| **Reduced Model, AIC = 27** | | | | 2 | **1E-06** |
| Stone status (case) | 1.2 | 0.22 | 5.2 | 1 | **1E-06** |
| **logUFe/Cre** | | | | | |
| **Full Model, AIC = 90** | | | | 6 | **0.2** |
| Stone status (case) | 1.4 | 0.37 | 3.7 | 1 | **0.0004** |
| Sex (male) | -0.42 | 0.34 | -1.2 | 1 | 0.2 |
| Breed | NA | NA | NA | | 0.8 |
| Age | 0.01 | 0.07 | 0.2 | 1 | 0.8 |
| Diet | | | | 2 | 0.06 |
| Hill's u/d | -1.2 | 0.49 | -2.4 | | **0.007** |
| Royal Canin SO | -0.47 | 0.54 | -0.9 | | 0.3 |
| **Reduced Model, AIC = 67** | | | | 4 | **0.001** |
| Stone status (case) | 1.1 | 0.29 | 3.8 | 1 | **0.0003** |
| Diet | | | | 2 | 0.2 |
| Hill's u/d | -1.2 | 0.41 | -2.9 | | **0.005** |
| Royal Canin SO | -0.39 | 0.45 | -0.88 | | 0.4 |
| **logUV/Cre** | | | | | |
| **Full Model, AIC = 135** | | | | 6 | 0.19 |
| Stone status (case) | 0.54 | 1.1 | 1.7 | 1 | 0.2 |
| Sex (male) | 0.44 | 0.42 | 1.0 | 1 | 0.3 |
| Breed | NA | NA | NA | | 0.5 |
| Age | 0.003 | 0.09 | 0.03 | 1 | 1.0 |
| Diet | | | | 2 | 0.4 |
| Hill's u/d | 0.76 | 0.61 | 1.2 | | 0.2 |
| Royal Canin SO | 0.40 | 0.66 | 0.60 | | 0.5 |
| **Reduced Model, AIC = 116** | | | | 2 | **0.004** |
| Stone status (case) | 0.99 | 0.34 | 3.9 | 1 | **0.004** |

**Table 4.** Regression and analysis of covariance (ANCOVA) results for a CaOx urolithiasis model with urinary variables and sex as predictors. The full model had a lower (better) AIC than any of the reduced models and thus is the only one reported. For variables with 1 degree of freedom, the status that corresponds to the estimate is in parentheses.

| Stone Status | Degrees of freedom | Estimate | Standard Error | T value | P-value |
|---|---|---|---|---|---|
| **Full Model, AIC = -191** | 5 | | | | **1E-09** |
| logUCa/Cre | 1 | 0.16 | 0.03 | 5.1 | **1E-06** |
| logUFe/Cre | 1 | 0.08 | 0.03 | 3.0 | **0.004** |
| logUV/Cre | 1 | 0.05 | 0.02 | 2.3 | **0.03** |
| Sex (male) | 1 | 0.27 | 0.09 | 3.1 | **0.002** |

**Table 5.** Simple linear regression results of *SLC39A10* genotype as a predictor of urinary calcium and trace metal-to-creatinine ratios in Miniature Schnauzer dogs. Values are reported in mg/g for UCa/Cre and µg/g for the urinary metal-to-creatinine ratios.

| Variable | Estimate | Standard Error | Raw p-value | Bonferroni-Holm p-value |
|---|---|---|---|---|
| **UCa/Cre** | **0.78** | **0.29** | **0.01** | **0.15** |
| **UZn/Cre** | -0.25 | 0.24 | 0.30 | 1 |
| **USr/Cre** | **0.77** | **0.33** | **0.03** | **0.30** |
| **UCd/Cre** | -0.36 | 0.29 | 0.22 | 1 |
| **UV/Cre** | 0.03 | 0.52 | 0.96 | 1 |
| **UCr/Cre** | -0.14 | 0.48 | 0.77 | 1 |
| **UFe/Cre** | -0.06 | 0.39 | 0.89 | 1 |
| **UMn/Cre** | 0.47 | 0.44 | 0.29 | 1 |
| **UCo/Cre** | -0.03 | 0.46 | 0.94 | 1 |
| **UNi/Cre** | 0.04 | 0.28 | 0.89 | 1 |
| **UCu/Cre** | -0.02 | 0.30 | 0.94 | 1 |
| **URb/Cre** | -0.09 | 0.17 | 0.58 | 1 |
| **UBa/Cre** | 0.02 | 0.33 | 0.96 | 1 |

**Figure 2.** Box and whisker plots of fasting spot urinary **A**) calcium-to-creatinine (UCa/Cre, mg/g) and **B**) strontium-to-creatinine (USr/Cre, µg/g) ratios in Miniature Schnauzer dogs that have a clear, carrier, or homozygous state for the *SLC39A10* haplotype that has previously been associated with risk for CaOx urolithiasis. The boxes represent the interquartile range ($25^{th}$ – $75^{th}$ percentile), the horizontal line within the boxes represents the median, and the whisker bars extend to 1.5 times the interquartile range. Circles represent outliers. Bonferroni-Holm corrected p-values are reported.

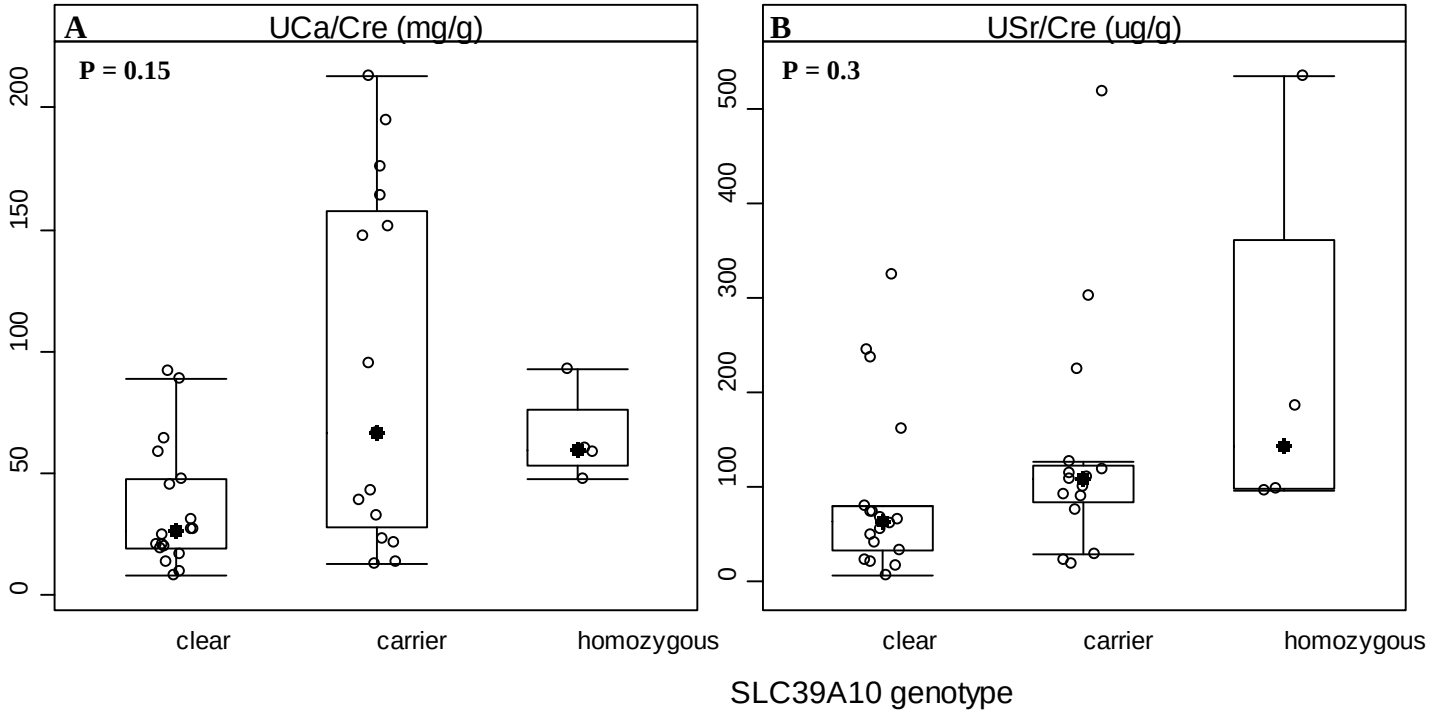

**Figure 3.** Correlation between log-transformed urinary calcium-to-creatinine (UCa/Cre) and strontium-to-creatinine (USr/Cre) ratios.

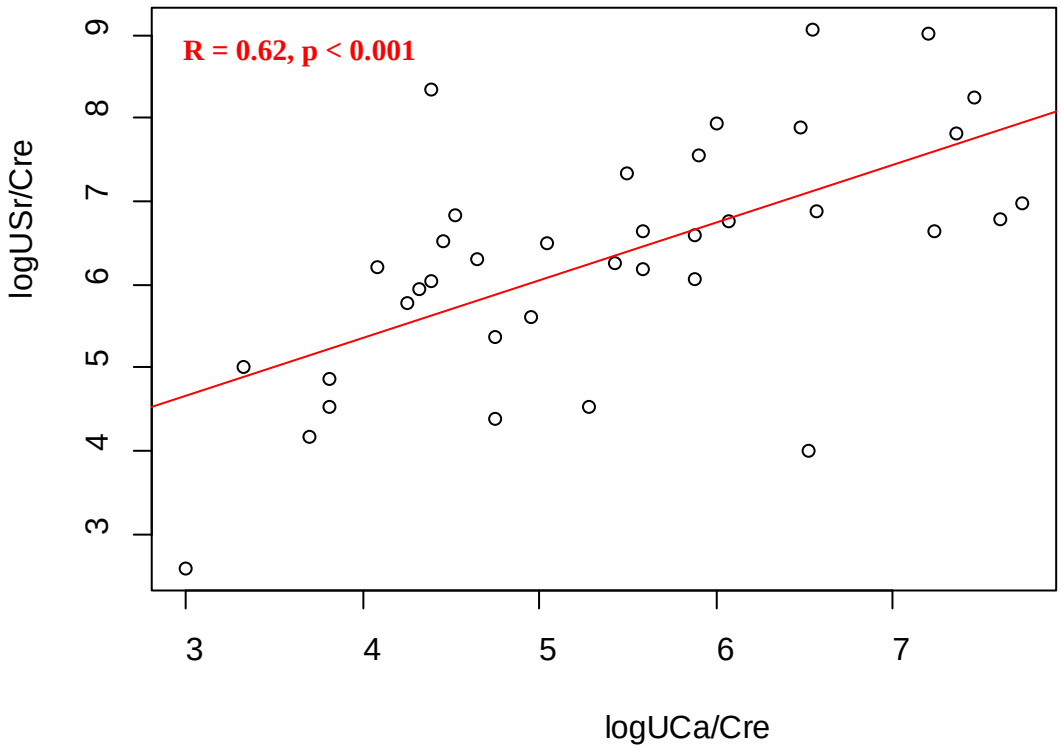

**Table 6.** Regression and analysis of covariance (ANCOVA) results for the effect of *SLC39A10* genotype (0, 1 or 2 copies of the risk haplotype) on log-transformed urinary calcium-to-creatinine (UCa/Cre) and strontium-to-creatinine (USr/Cre) ratios and on stone status. The reduced model represents the model with the best AIC as determined by stepwise selection of predictors. For variables with 1 degree of freedom, the status corresponding to the estimate is in parentheses.

| | Estimate | Standard Error | T value | Degrees of Freedom | P-value |
|---|---|---|---|---|---|
| **logUCa/Cre** | | | | | |
| **Full Model, AIC = 17** | | | | 7 | **0.05** |
| *SLC39A10* (risk haplotype) | 0.63 | 0.31 | 2.0 | 1 | **0.05** |
| Stone status (case) | 0.96 | 0.44 | 2.2 | 1 | **0.04** |
| Sex (male) | -0.26 | 0.44 | -0.60 | 1 | 0.6 |
| Age | 0.12 | 0.10 | 1.2 | 1 | 0.2 |
| Diet | | | | 2 | 0.6 |
| Hill's u/d | 0.61 | 0.90 | 0.68 | | 0.5 |
| Royal Canin SO | -0.50 | 0.93 | -0.53 | | 0.6 |
| **Reduced Model, AIC = 12** | | | | 3 | **0.006** |
| *SLC39A10* (risk haplotype) | 0.56 | 0.30 | 1.9 | 1 | 0.07 |
| Stone status (case) | 0.82 | 0.40 | 2.05 | 1 | **0.05** |
| **logUSr/Cre** | | | | | |
| **Full Model, AIC = 31** | | | | 7 | **0.05** |
| *SLC39A10* (risk haplotype) | 0.93 | 0.38 | 2.5 | 1 | **0.02** |
| Stone status (case) | 0.11 | 0.53 | 0.20 | 1 | 0.8 |
| Sex (male) | -0.50 | 0.53 | -0.95 | 1 | 0.4 |
| Age | 0.11 | 0.12 | 0.87 | 1 | 0.4 |
| Diet | | | | 2 | 0.8 |
| Hill's u/d | -0.74 | 1.1 | -0.68 | | 0.5 |
| Royal Canin SO | -0.48 | 1.1 | -0.43 | | 0.7 |
| **Reduced Model, AIC = 24** | | | | 2 | **0.03** |
| *SLC39A10* (risk haplotype) | 0.77 | 0.33 | 2.3 | 1 | **0.03** |
| **Stone status** | | | | | |
| **Full Model, AIC = 51** | | | | 3 | **0.05** |
| *SLC39A10* (risk haplotype) | 1.1 | 0.57 | 1.9 | 1 | **0.05** |
| Sex (male) | 1.0 | 0.83 | 1.2 | 1 | 0.2 |
| **Reduced Model, AIC = 50** | | | | 2 | **0.04** |
| *SLC39A10* (risk haplotype) | 1.2 | 0.56 | -2.1 | 1 | **0.04** |

# Chapter 7

# Conclusions and future directions

Part I: Disease and model importance

Nephrolithiasis is a common and painful condition that affects roughly 10% of the population (1). The efficacy of current preventative treatments is poor, and recurrence rates are high. The majority of kidney stones are composed of CaOx, and hypercalciuria is the most common metabolic abnormality underlying stone risk (10). The risk for CaOx stones is believed to be nearly equally divided between environmental and inherited factors. However, the specific environmental exposures and genes involved have been difficult to identify, and the prevalence of CaOx urolithiasis is increasing (8). Novel research approaches are needed to determine etiopathologic factors in stone development.

While most laboratory animals seem relatively resistant to forming CaOx stones, dogs naturally develop these stones. As with humans, canine CaOx stones are common, recurrent, and on the rise (150). Importantly, there is also an apparent inherited component to the disease in dogs, due to strong breed predispositions. Purebred dogs are well-suited to genetic studies and offer a new avenue for ascertaining susceptibility genes for CaOx urolithiasis. In people, it is estimated that multiple genes contribute to CaOx stone risk, and this complexity has hindered human genetic studies. In contrast, within an individual dog breed, genetic diseases are often caused by a small number of loci with strong effect, and LD is 10-100x that in people (62). Furthermore, other urolith types such as cystine and urate have shared susceptibility genes between dogs and people (71-76).

Part II: Research aims, findings, and future directions

The goal of this research was to identify metabolic and genetic risk factors for

urolithiasis. The first aim was to demonstrate that 2,8-DHA urolithiasis provides yet another example of stone disease with a shared susceptibility gene between dogs and people. Mutations in *APRT* cause 2,8-DHA urolithiasis in people, but the genetic basis of this urolith type had not previously been evaluated in dogs. In **Chapter 2**, a candidate gene approach was successfully used to identify an autosomal recessive *APRT* mutation in five NAIDs and one mixed breed dog with 2,8-DHA urolithiasis. Homozygosity for the missense mutation was strongly associated with disease. The mutation occurred at a highly conserved codon, and the identical missense mutation has been previously reported as a causal mutation for APRT deficiency in a human cell line (94). The results of this study emphasize the overlap in the genetic basis of inherited urolith disorders in dogs and people. It is hypothesized that one or more of the genetic risk factors for CaOx urolithiasis are also shared between these two species.

Prior to embarking on genetic studies of CaOx urolithiasis, urinary metabolites were measured in three dog breeds to determine which suffer from hypercalciuria and to ascertain if the trait is fixed within the breeds (**Chapter 3**). Miniature Schnauzers, Bichons Frise, and Shih Tzus with a history of CaOx urolithiasis were found to have higher urinary calcium than breed-matched, stone-free controls. Blood ionized calcium levels were also higher in stone-formers than controls, but no dog was hypercalcemic. Urinary oxalate levels did not differ by stone status. Based on this data, all three of the breeds are appropriate for case-control studies on the genetic basis of CaOx urolithiasis and IH. Though this research documented IH in three breeds, it did not determine the pathogenesis of IH. More extensive metabolic studies are needed to better characterize the nature of the disturbance that causes hypercalciuria in canine stone-formers. The

higher blood calcium levels in stone-formers raise suspicion for bone resorption or intestinal hyperabsorption of calcium as the source of hypercalciuria, but a concurrent renal resorption defect could also be present.

After the basic metabolic characteristics of canine CaOx urolithiasis had been assessed, the Miniature Schnauzer and Bichon Frise breeds were selected for GWAS to find disease-associated chromosomal loci (**Chapter 4**). In an initial analysis using Miniature Schnauzers, a strong association signal was found on CFA37. However, expansion of the study cohort altered the results such that the CFA37 hit was no longer significant. In an attempt to clarify the analyses, the population was pared down to the dogs with the best phenotypes and reanalyzed. The CFA37 locus remained in the background, but a new disease-associated locus emerged on CFA15. Analysis of a small group of Bichons Frise also found a disease-associated locus on CFA15, and a combined breed analysis strengthened the hit. Haplotype analysis revealed a risk haplotype that was present in most of the Bichon Frise stone-formers and a subset of the Miniature Schnauzers. This locus was independently associated with disease in both breeds. Surprisingly, the locus shared between the breeds was distinct from the CFA15 hit that occurred when Miniature Schnauzers were analyzed alone.

The GWAS findings suggest that CaOx urolithiasis is a polygenic disease in dogs. Within the Bichon Frise breed, there may be only one locus of major effect on CFA15. However, Miniature Schnauzers may have three loci that contribute to the inherited portion of stone risk. All three loci, the two on CFA15 and one on CFA37, encompass plasma membrane transporter genes. Validation of these risk loci in independent populations and sequencing of the positional transporter genes are indicated.

Identifying causal mutations for CaOx urolithiasis is not a simple task, even for a risk locus that has been narrowed down to a few Mb. While only 2% of the genome encodes protein, it is estimated that 80% or more has functional activity (178). Promoter elements typically reside within 1 kb of the transcriptional start site, but there are also distant *cis*-regulatory elements one or more Mb away from the target gene. Polymorphisms in *cis*-regulatory elements are estimated to account for 25-35% of the difference in gene expression between individuals (179).

Given the potential for a mutation to reside within regulatory regions and the knowledge that multiple risk loci likely exist for canine CaOx urolithiasis, a WGS approach was taken to evaluate for causal mutations in Miniature Schnauzers. This permits discovery of all variants, coding and non-coding, across the genome. Two CaOx urolithiasis cases and two controls were selected for sequencing based off their SNP haplotypes on CFA37 (the first locus found in the GWAS), and the WGS data for the CFA37 susceptibility locus was examined in **Chapter 5**. There were 18 positional candidate genes. Coding variants were identified in three genes, but they were deemed unlikely to be causal based on poor conservation within vertebrates and/or lack of predicted effect on protein function (e.g. synonymous SNPs). One positional gene, *SLC39A10*, stood out as a top candidate due to the importance of solute carriers in other inherited stone disease and its known expression in tissues involved in calcium homeostasis (kidney and intestines). A 3'UTR 16 bp duplication and an intronic 23 bp deletion were present in the case dogs. These variants were in strong LD, and follow-up genotyping in a large Schnauzer cohort revealed that neither was specific to the GWAS SNP risk haplotype on CFA37. As a result, the association between these variants and

disease was weak relative to other markers in the region. Other non-coding *SLC39A10* case variants included SNPs and small indels (≤5 bp).

A causal mutation has not yet been found at the Miniature Schnauzer CFA37 risk locus, but three important points need to be made in interpreting the WGS results. First, there is a gap in the canine reference where the promoter region for *SLC39A10* is predicted to lie. As a result, this region could not be evaluated. The reference sequence for the missing promoter region is going to be obtained by sequencing canine bacterial artificial clones. Second, even simple SNPs in non-coding regions can be sufficient to cause disease. Classic examples are intronic SNPs in osteoporosis and Hirschprung's disease and promoter SNPs in β-thalassemia, hemophilia B, and familial hypercholesterolemia (180). Thus, one of the non-coding SNPs or small indels in *SLC39A10* could be the causative mutation. Finally, coding sequence for another gene in the region, *PLCL1*, could not be obtained. This gene plays a role in intracellular calcium signaling and cannot be ruled out as a stone susceptibility gene.

More research is needed to search for a causal mutation for canine CaOx urolithiasis on CFA37 and complete the assessment of *SLC39A10* and *PLCL1* as positional candidate genes. The missing sequence for both genes must be obtained. Once all candidate variants have been annotated, a mass array will be designed to rapidly genotype a large population of dogs for candidate variants and determine which are most strongly associated with disease. Copy number variant analyses will also be performed for the CFA37 region to uncover larger structural variants associated with disease. After variant discovery is complete, studies will be designed to confirm a functional effect of the top variant(s). To determine if dogs with the variant(s) have abnormal gene

expression, qRT-PCR (with or without relative allelic quantification) will be performed to compare RNA levels between cases with the variant and clear controls. Western blot analysis and immunohistochemistry will help determine if there are differences in protein expression or localization. *In vitro* approaches will also be designed to test the effect of specific variants on gene expression by using a synthetic reporter construct.

The aforementioned studies test the effect of variants on gene expression, but the link between the gene and disease would still need to be established. A unique approach to investigate the role of *SLC39A10* in CaOx urolithiasis is offered through a recently established fly model. The fly Malpighian tubules function analogous to human nephrons, with many homologous proteins and transport systems. The *Drosophila* system lends itself to both physical and genetic manipulation (181), and there is a *Drosophila* model where CaOx crystal formation develops within 1-2 hrs exposure of the tubules to oxalate and within days of dietary oxalate ingestion (182, 183). This fly model has been used to demonstrate that Slc26a5/6 is a potent oxalate transporter, and knockdown reduces CaOx microlith formation (182). In the fly, the expression of the *SLC39A10* homolog, CG10006, is highly specific to the renal tubules during both larval and adult stages (184). Inhibitory RNA fly lines can be combined with a urate oxidase promoter/Gal4 driver system to create *SLC39A10* renal-specific knockdowns. The knockdown flies could then be compared to wild type flies to identify differences in speed of formation, quantity, or composition of crystals. These *SLC39A10* fly experiments are currently underway in collaboration with the Romero Transporter Physiology Laboratory at Mayo Clinic.

During the process of considering *SLC39A10* as a candidate gene, new questions arose about factors that influence stone risk. The protein encoded by *SLC39A10* is a metal ion transporter with affinity for Zn and Cd. High dietary Zn intake has been associated with increased risk for kidney stones in people (146, 147), and Zn in the most abundant metal in CaOx stones (144). Cd is an environmental toxin that is linked to CaOx nephrolithiasis even at relatively low levels of exposure (153). Disturbances in metal uptake or elimination from body could occur with *SLC39A10* polymorphisms and serve as the mechanism behind increased stone risk. Metals could also be a general environmental risk factor for stones, independent of *SLC39A10*. The role of metals in CaOx urolithiasis had not previously been explored in dogs and could have important implications in dietary formulation and in examining *SLC39A10* as a urolithiasis candidate gene.

In **Chapter 6**, two approaches were taken to investigate metals in stone disease. The first aim was to determine if there was a relationship between urinary trace metals and CaOx stone status in multiple breeds. The second aim was to test for a relationship between urinary trace metals and *SLC39A10* genotype in Miniature Schnauzer dogs.

The results of the urinary metals experiments were unexpected but note-worthy. Neither urinary Zn nor Cd had a relationship to stone status or the *SLC39A10* genotype. However, urinary V and Fe were higher in case dogs of multiple breeds compared to stone-free controls, and urinary Sr was higher in Miniature Schnauzers with the *SLC39A10* stone risk haplotype than breed-matched dogs clear of the haplotype. Both V and Fe can affect bone turnover and could theoretically increase bone calcium release and cause a secondary hypercalciuria. Alternatively, these metals could be biomarkers of a

bone resorption or intestinal hyperabsorption disorder. The high urinary V and Fe levels were noted in stone-formers of a variety of breeds, which suggests that they may be due to a shared environmental exposure. However, a common genetic risk factor cannot be ruled out. Serum and stone metal quantification and detailed dietary analysis would be helpful in teasing out the role of trace metals in CaOx urolithiasis.

The *SLC39A10* risk haplotype was associated with higher urinary Sr and calcium levels. Calcium and Sr are regulated by many of the same transporters and channels, and Sr loading is used to study disorders in calcium metabolism (175, 176). Zip10, the protein encoded by *SLC39A10*, has not been tested for Sr transport, but the positive relationship between the *SLC39A10* genotype and urinary Sr suggests that it could have affinity for this metal. Whether Sr has a direct or indirect role in lithogenesis or is simply a marker of abnormal calcium handling remains to be determined. The link between urinary measurements and *SLC39A10* genotype supports that there may be a functional mutation in this gene. However, it is also possible that the urinary abnormalities are caused by a mutation in another gene that is in LD with the *SLC39A10* haplotype. The fly knockdown experiments described above are needed to better elucidate whether *SLC39A10* is involved in stone risk.

Part III: Final conclusions

This research used the dog as a spontaneous model to investigate metabolic and genetic risk factors of CaOx urolithiasis. Three canine breeds were established to suffer from IH, a trait underlying stone risk in people. Genetic studies in two of the IH breeds identified three putative susceptibility loci for CaOx urolithiasis. Genotype for a

positional solute carrier gene, *SLC39A10*, was found to correlate with urinary Sr and calcium levels, as well as stone risk. This gene is a novel candidate gene for CaOx urolithiasis. With future sequencing and expression studies, our goal is to find one or more mutations that impart risk for IH and CaOx stone development. It is predicted that canine CaOx urolithiasis susceptibility genes will also impact human stone disease. The dog can also serve as a model for exploring environmental risk factors such as trace metals and gene-environment interactions that contribute to stone pathogenesis.

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# Appendix

*Permission for Inclusion of Published Work*

One of the chapters in this dissertation has been published, and only minor modifications were made to conform to formatting requirements for the dissertation:

**Chapter 2 –** An *APRT* mutation is strongly associated with and likely causative for 2,8-dihydroxyadenine urolithiasis in dogs. *Mol Genet Metab* 2014;111:399-403.

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