Molecular Genetics and Metabolism Reports 29 (2021) 100792

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: www.elsevier.com/locate/ymgmr

Multiple variants in *XDH* and *MOCOS* underlie xanthine urolithiasis in dogs

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ARTICLE INFO

Keywords:
Xanthine dehydrogenase
Molybdenum cofactor sulfurase
Hereditary xanthinuria
Canine genetics
Kidney stones
Nephrolithiasis

ABSTRACT

Hereditary xanthinuria is a rare autosomal recessive disease caused by missense and loss of function variants in the xanthine dehydrogenase (*XDH*) or molybdenum cofactor sulfurase (*MOCOS*) genes. The aim of this study was to uncover variants underlying risk for xanthinuria in dogs. Affected dogs included two Manchester Terriers, three Cavalier King Charles Spaniels, an English Cocker Spaniel, a Dachshund, and a mixed-breed dog. Four putative causal variants were discovered: an *XDH* c.654G > A splice site variant that results in skipping of exon 8 (mixed-breed dog), a *MOCOS* c.232G > T splice site variant that results in skipping of exon 2 (Manchester Terriers), a *MOCOS* p.Leu46Pro missense variant (Dachshund), and a *MOCOS* p.Ala128Glyfs*30 frameshift variant that results in a premature stop codon (Cavalier King Charles Spaniels and English Cocker Spaniel). The two splice site variants suggest that the regions skipped are critical to the respective enzyme function, though protein misfolding is an alternative theory for loss of function. The *MOCOS* p.Leu46Pro variant has not been previously reported in human or other animal cases and provides novel data supporting this residue as critical to MOCOS function. All variants were present in the homozygous state in affected dogs, indicating an autosomal recessive mode of inheritance. Allele frequencies of these variants in breed-specific populations ranged from 0 to 0.18. In conclusion, multiple diverse variants appear to be responsible for hereditary xanthinuria in dogs.

1. Introduction

Hereditary xanthinuria is a rare autosomal recessive disease characterized by excessive xanthine, a metabolic by-product of purine metabolism, in the urine [1,2]. Due to its low solubility, xanthine can precipitate out in the urine as xanthine crystals and lead to the formation of urinary stones and secondary renal injury [1,2]. At this time, there is no specific cure for xanthinuria, but it is recommended that patients consume a low-purine diet and increase fluid intake to reduce risk for stone formation [1].

Xanthine dehydrogenase (XDH) is an enzyme in the purine metabolism pathway that catalyzes the conversion of both hypoxanthine to xanthine and xanthine to uric acid [1,2]. Molybdenum cofactor sulfurase (MOCOS) converts the molybdenum cofactor of XDH from the oxo-form to the sulfide- form, which is necessary for XDH activity [2,3]. Individuals with xanthinuria type I have *XDH* variants that result in an XDH deficiency (OMIM #278300) [4], while individuals with xanthinuria type II have *MOCOS* variants that result in both MOCOS and XDH deficiencies (OMIM #603592) [5]. Clinically, the subtypes of hereditary xanthinuria are indistinguishable [2].

Hereditary xanthinuria is suspected to occur in Cavalier King Charles Spaniel [6–9] and Dachshund dogs [10–12], but genetic investigations are lacking with no underlying variants reported to date. The aim of this study was to characterize the molecular basis of hereditary xanthinuria in eight dogs naturally affected by the disease. The affected dogs comprised four dog breeds (Manchester Terrier, Cavalier King Charles Spaniel, English Cocker Spaniel, and Dachshund) and a mixed-breed dog. We identified four pathogenic variants, one in *XDH* and three in *MOCOS*, including two in-frame exon skipping variants which were confirmed with cDNA analysis. The variant locations and effects

Abbreviations: XDH, xanthine dehydrogenase; MOCOS, molybdenum cofactor sulfurase; OMIM, Online Mendelian Inheritance in Man; MUC, Minnesota Urolith Center; FFPE, formalin-fixed paraffin-embedded; DBVDC, Dog Biomedical Variant Database Consortium; MX, mixed breed; MT, Manchester Terrier; CKCS, Cavalier King Charles Spaniel; ECS, English Cocker Spaniel; DACH, Dachshund.

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https://doi.org/10.1016/j.ymgmr.2021.100792
Received 16 August 2021; Accepted 19 August 2021

contribute to the understanding of domains critical to XDH and MOCOS protein function.

2. Materials and methods

2.1. Animals – xanthine urolithiasis cases

A search of submissions to the Minnesota Urolith Center (MUC) between 2010 and 2014 was performed to identify canine xanthine uroliths, defined as those with a stone layer composed of >70% xanthine as determined by infrared spectroscopy and polarizing light microscopy. Only samples submitted from addresses within North America were considered. Dogs with xanthine urolithiasis but without a history of receiving allopurinol (an XDH inhibitor) or a previous history of urate uroliths were presumed to be cases of hereditary xanthinuria. Veterinary clinics of the dogs with presumed hereditary xanthinuria were contacted to request owner participation in the study. Owners of eight dogs with xanthine urolithiasis agreed to participate, including six dogs from the 2010–2014 MUC population and 2 additional dogs with xanthine uroliths whose owner or veterinarian contacted the University of Minnesota Canine Genetics Laboratory due to suspicion for hereditary xanthinuria (one whose uroliths were analyzed at the MUC after the database search timeframe and the other whose uroliths were analyzed at the Canadian Veterinary Urolith Centre, University of Guelph). The hereditary xanthinuria study population comprised three Cavalier King Charles Spaniels, two Manchester Terriers, one English Cocker Spaniel, one Dachshund, and one mixed-breed dog. Whole blood, cheek swabs, and FFPE tissue (from a deceased dog) were submitted from the cases for DNA extraction.

2.2. Animals – breed populations

Breed populations were established for genetic comparisons and comprised 109 Cavalier King Charles Spaniels, 386 Manchester Terriers, 285 English Toy Terriers, 116 Dachshunds, and 42 English Cocker Spaniels. None of the dogs in the breed populations had a reported history of xanthine urolithiasis, but the dogs did not undergo stone screening procedures. No restrictions on gender, age, or relatedness were placed. DNA samples from the breed populations were obtained from owner and veterinarian submissions of EDTA blood and cheek swabs for diagnostic testing (Manchester Terriers), banked DNA at the Canine Health Information Center (CHIC) database at the University of Missouri (Manchester Terriers), banked DNA at the University of Minnesota (UMN) Canine and Feline Genomics Laboratory (Cavalier King Charles Spaniels and Dachshunds), and banked DNA at the University of Missouri (English Cocker Spaniels).

The variant catalogue of the Dog Biomedical Variant Database Consortium (DBVDC) was used to determine variant frequencies in a large diverse population [13]. This catalogue currently comprises of whole genome sequencing variant calls from 804 dogs from >140 breeds and 9 wolves.

Written owner consent was obtained for all prospectively recruited samples, and the study protocol (#1509-33019A) was approved by the University of Minnesota Institutional Animal Care and Use Committee.

2.3. Pedigree analysis

Pedigrees were obtained from Manchester Terrier owners and breeders and through online databases (World Pedigree and the American Kennel Club). Genial Pedigree Draw (Genial Genetic Solutions ltd, Chester, UK) was used to create a pedigree to visualize relationships between cases. Pedigree analysis was not performed for the other dog breeds, as pedigrees for the cases were not available.

2.4. DNA sequencing and analysis

For prospectively recruited samples, genomic DNA was extracted from whole blood, buccal swabs, or FFPE tissue using the standard protocol for Gentra Puregene Blood Kit (Qiagen Sciences, Germantown, MD, USA). Primer3 was used to design primers to examine the exonic sequence of *XDH* and *MOCOS* (Supplementary Table 1 & 2) [14]. Sanger sequencing of the 36 exons of *XDH* (ENSCAFT00000047701.2) and the 15 exons of *MOCOS* (ENSCAFT00000028243.4) was performed for four of the dogs with xanthine urolithiasis (one for each breed except the English Cocker Spaniel) and compared to the canine reference genome (CanFam3.1). Sequencher 5.1 DNA Sequence Analysis Software was used to identify variants unique (not present in the reference genome or in any of the sequenced controls) to our cases (Supplementary Table 3) (Gene Codes Corporation, Ann Arbor, MI, USA). Two of the breeds, Manchester Terriers and Cavalier King Charles, had more than one case; only one case was sequenced for all the exons, while the other cases were only sequenced for identified variants. The English Cocker Spaniel case was only sequenced for exons containing the putative causal variants discovered in the other four breeds. Since the dog was discovered to have one of these variants, sequencing of the remaining exons was not performed. DNA from the English Cocker Spaniel was isolated from FFPE tissues and was thus lower quality, so primers were designed to give shorter products around the putative causal variants and are described in Supplementary Table 4.

2.5. Variant prioritization

Genomic positions of variants were based on the canfam3.1 dog reference genome. Unique variants were prioritized based on predicted functional effect, conservation, and frequency in the breed populations and the DBVDC. Functional effect was predicted via two variant pathogenicity prediction programs. Missense variants were first analyzed via Ensembl Variant Effect Predictor (VEP), which utilizes SIFT to give scores based on alignment to protein family and type of amino acid change [15,16]. Variants were also analyzed with MutPred2, a tool which uses a random forest model to predict the effect of, and thus, infers the pathogenicity of, missense variants on protein structure and function [17]. InterProScan, an online tool which classifies proteins into families and predicts the location of domains and important sites, was used to determine if putative causal variants were present in protein domains or other important sites [18].

Conservation scores for the putative causal variants were determined from the "100 Vertebrates Basewise Conservation by phyloP (phyloP100way)" track on the UCSC Genome Browser [19]. The phyloP100way scores are the –log10(*p*-value) for rejecting the null hypothesis of neutral evolution; positive scores indicate conservation, and negative scores indicate acceleration. The "Vertebrate Multiz Alignment & Conservation (100 Species)" track was used to determine the number of species the mutated amino acid was conserved across [20].

2.6. Variant genotyping assays

Genotyping assays were developed to estimate variant frequencies within breed populations. NEBcutter V2.0 was used to identify restriction enzyme sites for commercially available enzymes [21]. If a restriction enzyme site was identified that distinguished the reference from variant sequence, a restriction fragment length polymorphism (RFLP) was used for genotyping. If no restriction enzyme site was identified, allele specific PCR was used for genotyping. Samples from one dog of each genotype, for each breed, were directly sequenced with standard Sanger sequencing for each putative causal variant and used as genotype controls for all assays. Control primers for another gene were included in the allele specific assays for added assurance of results. Assay type, primers, and conditions are described in Supplementary

Table 5.

2.7. cDNA synthesis and sequencing

Blood was collected in Tempus™ Blood RNA Tubes (Thermo Fisher Scientific Inc., Waltham, MA, USA), and RNA was extracted using the standard protocol from the Tempus™ Spin RNA Isolation Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). cDNA was synthesized using the standard protocol for SuperScript® First-Strand Synthesis System (Thermo Fisher Scientific Inc., Waltham, MA, USA). For splice site variants, primers were designed to amplify a product spanning from the exon before to the exon after the exon containing the putative causal variant. cDNA primers for the Manchester Terriers and the mixed-breed dog were designed using Primer 3 (primers and conditions described in Supplementary Table 6) [14].

2.8. Additional phenotyping

Urine samples were collected from two females and one male Manchester Terriers from the breed population after they were discovered to be homozygous for the putative causal variant discovered in the cases. The urine from the male dog was instead analyzed by infrared spectroscopy for xanthine crystalluria. The urine from the female dogs was submitted for xanthine quantification and compared to a healthy twelve-year-old female mixed-breed dog that had no copies of any of the putative causal variants. Urine xanthine quantification, run in triplicate, was determined by liquid chromatography tandem mass spectrometry at the Center for Mass Spectrometry and Proteomics, University of Minnesota.

3. Results

3.1. Animals – submissions to Minnesota Urolith Center

Between 2010 and 2014, the MUC received 70 canine xanthine stone submissions, 53 (76%) of which were determined to be from iatrogenic causes (dogs confirmed or assumed to be receiving the XDH inhibitor, allopurinol, due to a previous history of urate uroliths or for treatment of Leishmaniasis). The remaining 17 stones (24%) were presumed to be caused by hereditary xanthinuria. Some dogs had multiple stone episodes (recurrences) during the study time frame, thus the 70 stones came from 62 dogs. Breed totals, with duplicate submissions removed, for iatrogenic and presumed hereditary cases are listed in Table 1.

3.2. Phenotypic description of xanthine urolithiasis cases

Eight dogs with presumed hereditary xanthinuria were available for study participation, including 6 from the 2010–2014 MUC population and 2 additional dogs with xanthine uroliths whose owner or veterinarian contacted the University of Minnesota Canine Genetics Laboratory due to suspicion for hereditary xanthinuria. The population comprised 2 Manchester Terriers, 3 Cavalier King Charles Spaniels, 1 English Cocker Spaniel, 1 Dachshund and 1 mixed-breed dog. Five dogs were male, and three were female. Age at first diagnosis ranged from 7 weeks to 4 years. Additional signalment characteristics and clinical presentations are reported in Table 2.

3.3. DNA sequencing and analysis

Sanger sequencing of *XDH* and *MOCOS* identified four putative causal variants for hereditary xanthinuria (Table 3): two variants affected splicing and resulted in the complete removal of exons (in-frame); one was a deletion resulting in a frameshift and the formation of a premature stop codon; and the last was a missense variant. None of the variants were present in the DBVDC. Following the human terminology, we classified the putative causal variant in *XDH* as Xanthinuria Type I and the putative causal variants in *MOCOS* as Xanthinuria Type II.

Table 1
Breed counts and etiology for dogs with xanthine uroliths submitted to the Minnesota Urolith Center between 2010 and 2014.

Iatrogenic		
	Breed	#
77% (48/62)	Dalmatian	27
	English Bulldog	5
	Mixed-breed	5
	Dachshund	1
	Italian Spinone	1
	American Bulldog	1
	Cavalier King Charles Spaniel	1
	Cocker Spaniel	1
	Golden Retriever	1
	Jack Russell Terrier	1
	Norfolk Terrier	1
	Olde English Bulldogge	1
	Pomeranian	1
	Shih tzu	1
	Total Number of Dogs	48
Hereditary		
	Breed	**#**
23% (14/62)	Cavalier King Charles Spaniel	5
	English Cocker Spaniel	2
	Dachshund	2
	Mixed-breed	3
	Chihuahua	1
	Manchester Terrier	1
	Total Number of Dogs	**14**

Table 2
Signalment and clinical presentation for eight dogs with hereditary xanthine urolithiasis.

Dog	Breed	Sex	Clinical Presentation
1	Manchester Terrier	Intact male	Urethral obstruction and cystoliths at 7 weeks of age
2	Manchester Terrier	Intact male	Uroliths at 2 yo (location within urinary tract not reported)
3	Cavalier King Charles Spaniel	Spayed female	Uroliths at 3.4 yo and 4.8 yo (location within urinary tract not reported)
4	Cavalier King Charles Spaniel	Neutered male	Urethral obstruction and cystoliths at 1 yo; cystoliths, renal mineralization, and chronic kidney disease at 2 yo; urethral obstruction at 4 yo; passed away from chronic kidney disease at 8 yo
5	Cavalier King Charles Spaniel	Intact male (neutered at time of recurrence)	Urethral obstruction, cystoliths, and renal mineralization diagnosed at 5 mos of age with recurrence; urethral obstruction, cystoliths, nephroliths, ureteroliths, renal mineralization, and chronic kidney disease at 1 yo
6	English Cocker Spaniel	Spayed female	Uroliths at 4 yo (location within urinary tract not reported)
7	Mixed-breed	Neutered male	Urethral obstruction and renal mineralization at 2 yo
8	Dachshund	Spayed female	Nephrolith (4.5 cm) at 3 yo

3.3.1. Mixed-breed dog

The mixed-breed dog with xanthine uroliths was homozygous for a putative causal variant of the last base pair (bp) in exon 8 of *XDH*: c.654G > A, p.Leu218Leu (Table 3). The affected nucleotide is highly conserved (phyloP = 6.5) in 83 vertebrate species [19,20]. Ensembl VEP reported the consequence of the variant to be a synonymous, splice

Table 3
Putative causal variants for canine xanthinuria identified in *XDH* and *MOCOS*.

Gene – Classification	***XDH* - Type I**	***MOCOS* - Type II**		
Exon	8	2	4	1
Variant	c.654G > A p.Leu218Leu	c.232G > T p.Gly78Cys	c.383delC p.Ala128Glyfs*30	c.137 T > C p.Leu46Pro
Genomic Position	chr17:24,970,436	chr7:53,964,250	chr7: 53,959,096	chr7:53,971,022
Type of Mutation	Synonymous; Splicing	Missense; Splicing	Frameshift	Missense
SIFT	–	0	–	0.05
MutPred2	–	0.77	–	0.96
DBVDC Frequency	0	0	0	0
Breed	MX	MT	CKCS	ECS	DACH
Breed Frequency	–	0.13, $n = 387$	0.03, $n = 109$	0, $n = 42$	0, $n = 116$

Nucleotide and residue location determined on *XDH* transcript ENSCAFT00000047701.2 and the *MOCOS* transcript ENSCAFT00000028243.4. Genomic positions determined based on canfam3.1. Ensembl Variant Effect Predictor (outputs SIFT scores) and MutPred2 was used to predict pathogenicity for missense variants [15–17]. SIFT scores range from 0 to 1 with <0.05 predicted to be deleterious. MutPred2 scores range from 0 to 1 with >0.5 predicted to be deleterious. MX = mixed breed, DACH = Dachshund, MT = Manchester Terrier, CKCS = Cavalier King Charles Spaniel, ECS = English Cocker Spaniel.

region variant [15]. Splicing defects were verified through cDNA sequencing which revealed that the putative causal variant results in the removal of all 93 bp (31 amino acids) of exon 8 (p.Arg189_Leu218del) (Fig. 1). PhyloP scores determined the skipped exon contained highly conserved amino acids [19]. InterProScan predicted that the removed amino acids are part of the XDH small subunit (IPR014307, amino acids 6–523) [18]. The small subunit contains two Fe/S domains and a FAD domain and is involved in oxidation-reduction processes (GO:0055114), XDH activity (GO: 0004854), xanthine oxidase activity (GO:0004855), and flavin adenine dinucleotide binding (GO:0050660) [18].

Twelve other variants were identified in the mixed breed that were also unique to cases: 8 variants in *XDH* (7 present in a homozygous state and 1 heterozygous) and 4 variants in *MOCOS* (all present in a heterozygous state; Supplementary Table 3). Ten were determined to be synonymous, two were missense variants, and none were predicted to affect splice regions. Ensembl VEP reported the SIFT score for one missense variant, *XDH* p.Glu133Asp, to be benign/tolerated and the other, *MOCOS* p.Pro660Arg, to be deleterious [15]. MutPred2 predicted both missense variants to be benign [17]. The variant frequencies in the DBVDC were 0.28 for *XDH* p.Glu133Asp and 0.07 for *MOCOS* p.Pro660Arg. Given the *MOCOS* p.Pro660Arg variant's conflicting pathogenicity predictions and relatively low frequency in the DBVDC, its effect was deemed underdetermined. However, it was considered unlikely the cause of diseases in this dog because of the heterozygous genotype and the stronger evidence for *XDH* c.654G > A as the causal variant. The other 11 variants were considered neutral and not the cause of disease.

3.3.2. *Manchester Terriers*

The two Manchester Terriers with xanthine uroliths were homozygous for a putative causal variant of the last bp in exon 2 of *MOCOS*: c.232G > T, p.Gly78Cys (Table 3). This missense variant exchanges glycine, a nonpolar amino acid, for cysteine, a polar amino acid with a thiol group. The affected nucleotide is highly conserved (phyloP = 6.8) in 74 vertebrate species [19,20]. Ensembl VEP determined the consequence of the variant to be a missense, splice region variant and gave a SIFT score of 0 (deleterious) [15,16]. MutPred2 predicted the variant to be disease causing (score = 0.77) with the following molecular mechanisms of pathogenicity: altered metal binding (probability = 0.37, $p = 9.7 \times 10^{-3}$), altered ordered interface (probability = 0.32, $p = 8.7 \times 10^{-3}$), gain of an allosteric site at H81 (probability = 0.20, $p = 0.04$), and gain of catalytic site at N79 (probability = 0.13, $p = 0.03$) [17]. Splicing defects were verified through cDNA sequencing which revealed that the variant results in the removal of all 90 bp (30 amino acids) of exon 2 (p.Gly48_Tyr77del) (Fig. 2). PhyloP scores determined the skipped exon contained highly conserved nucleotides [19]. The

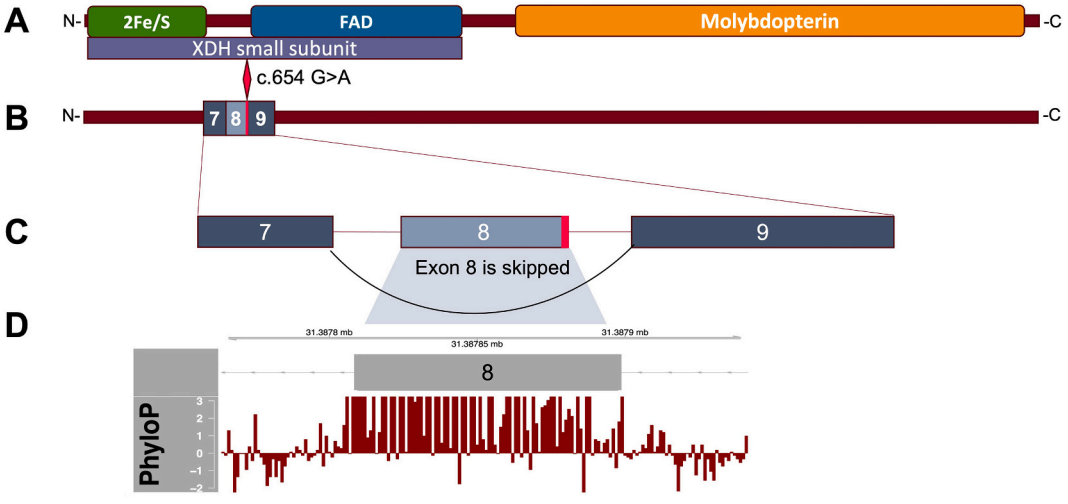

Fig. 1. Schematic of the effect of the mixed-breed Xanthinuria Type 1 variant. A) Location of the protein domains of xanthine dehydrogenase (XDH), including two Fe/S domains, a FAD domain, and a molybdopterin domain [2]. The putative causal variant for xanthinuria (in red) resides in the XDH small subunit, which spans the Fe/S and FAD domains. B) Exons 7–9 of the canine *XDH* transcript (ENSCAFT00000047701.2) are shown. The variant alters the last base pair of exon 8 and C) results in skipping of the exon. D) Plot demonstrating high conservation of the nucleotides across exon 8. Conservation is determined by phyloP100 way score; scores reflect the –log(p) with >3 corresponding to a *p*-value of <0.001 for rejecting the null hypothesis that the base substitution rate is consistent with neutral evolution.

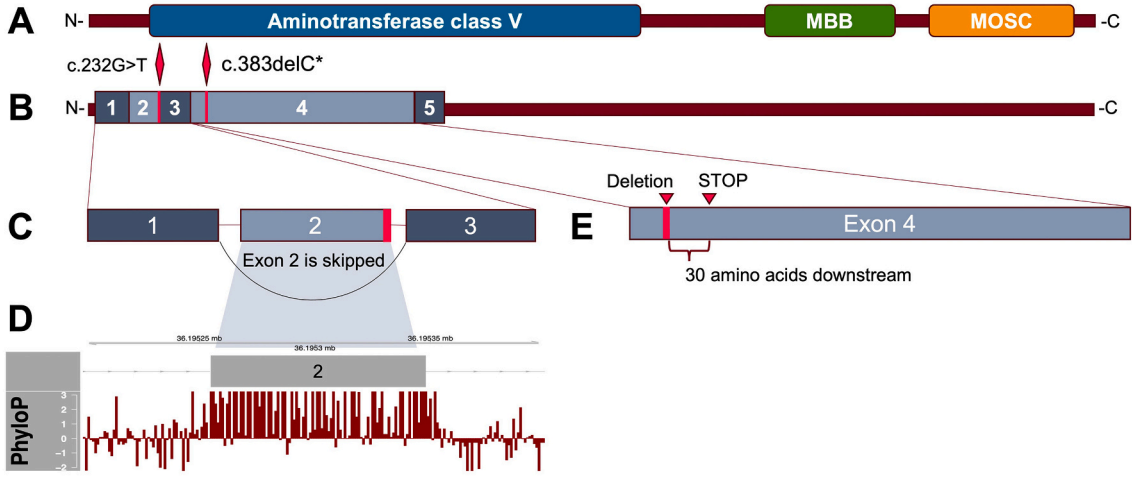

Fig. 2. Schematic of the effect of the Manchester Terrier, Cavalier King Charles Spaniel, and English Cocker Spaniel Xanthinuria Type 2 variants. A) Location of protein domains of molybdenum cofactor sulfurase (MOCOS), including the aminotransferase class V, MOSC N-terminal beta barrel (MBB), and MOSC domains. The putative causal variants for xanthinuria (in red) reside in the aminotransferase class V domain. B) Exons 1–5 of the canine MOCOS transcript (ENSCAFT00000028243.4). C) The c.232G > T variant alters the last base pair of exon 2 and results in exon skipping. D) Plot demonstrating high conservation of the nucleotides across exon 2. Conservation is determined by a phyloP100way score; scores reflect the –log(p) with >3 correspond to $p < 0.001$ for rejecting the null hypothesis that the base substitution rate is consistent with neutral evolution. E) The c.383delC* frameshift variant results in the formation of a premature stop codon 30 amino acids (89 bp) downstream in exon 4.

removed amino acids are part of the aminotransferase class-V protein domain (IPR000192, amino acids 50–481) [18].

Nine other variants were identified in the Manchester Terriers that were unique to the sequenced cases: 8 variants in *XDH* (4 present in a homozygous state and 4 heterozygous) and 1 *MOCOS* (present in a homozygous state; Supplementary Table 3). Eight of the nine variants were synonymous. While one was predicted to be a splice region variant, it was present in the DBVDC at an allele frequency of 0.27 and was considered likely neutral. A single missense variant in *XDH* (p.Glu133Asp) was identified in one of the Manchester Terrier cases and was present in the heterozygous state. The *XDH* p.Glu133Asp variant was predicted by Ensembl VEP and MutPred2 to be benign [15,17], and was present in the DBVDC at a frequency of 0.28. Thus, all nine variants were considered to be neutral in effect.

3.3.3. Cavalier King Charles and English Cocker Spaniels

The Cavalier King Charles Spaniels with xanthine uroliths were homozygous for a putative causal variant in exon 4 of *MOCOS*: c.383delC, p.Ala128GlyfsX30 (Table 3). The affected nucleotide is part of the aminotransferase class V domain of MOCOS (Fig. 2) [18]. The deletion results in a frameshift forming a premature stop codon 89 bp (30 amino acids) downstream. This results in a truncated protein (>80% loss) and in the loss of a large portion of the aminotransferase class V domain, the MOSC N-terminal beta barrel domain (IPR005303, amino acids 583–700), and the molybdenum cofactor sulfurase C terminal domain (IPR000192, amino acids 705–865) [18].

Nine other variants were identified in the Cavalier King Charles Spaniels that were unique to the sequenced case: 8 variants in *XDH* (6 present in a homozygous state and 2 heterozygous) and 1 variant in *MOCOS* (present in a homozygous state; Supplementary Table 3). Of these, 7 were synonymous variants with no predicted effect on splice regions. The two missense variants were both identified in *XDH*. One was *XDH* p.Glu133Asp; as discussed above, this variant was deemed likely neutral due to Ensembl VEP and MutPred2 predications and its frequency in the DBVDC database. The other was *XDH* p.Phe68Val, present in a heterozygous state. This variant was predicted to be benign/tolerated by Ensembl VEP but deleterious by MutPred2 [15,17]. However, it was considered likely neutral due to a relatively high frequency of 0.19 in the DBVDC. Based on this data, all 9 variants were considered likely neutral and not the cause of disease.

The English Cocker Spaniel DNA sample was obtained from *formalin-fixed, paraffin-embedded (FFPE)* tissue after the genetic analyses had been performed on the other cases and was genotyped for all exons containing the putative casual variants identified in the other xanthinuria cases and not present in controls. The case was homozygous for the same putative causal variant as the Cavalier King Charles Spaniels, *MOCOS* c.383delC (Table 3 and Fig. 2), as well as the likely benign variant *MOCOS* c.384G > A (Supplementary Table 3). The English Cocker Spaniel case had no copies of the Manchester Terrier or mixed-breed variants. Due to insufficient DNA quantity, we were unable to test for the Dachshund variant.

3.3.4. Dachshund

The Dachshund with xanthine uroliths was homozygous for a missense variant in exon 1 of *MOCOS* (c.137 T > C; p.Leu46Pro) (Table 3). The affected nucleotide is highly conserved (phyloP = 2.0), as are the others that make up the codon (phyloP = 1.2 and 1.1), in 53 vertebrate species [19,20]. Both leucine and proline are non-polar amino acids; however, proline contains a pyrrolidine, a cyclic amine, side chain. Ensembl's VEP predicted the p.Leu46Pro variant to be deleterious (SIFT score = 0.05), as did MutPred2 (score = 0.96) [15,17]. MutPred2 predicted the following molecular mechanisms of pathogenicity: gain of intrinsic disorder (probability = 0.33, $p = 0.03$), altered ordered interface (probability = 0.28, $p = 0.05$), and loss of helix (probability = 0.27, $p = 0.04$) [17].

Ten other variants were identified in the Dachshund that were unique to the sequenced case: 8 variants in *XDH* (4 present in a homozygous state and 4 heterozygous) and 2 variants in *MOCOS* (both present in a homozygous state; Supplementary Table 3). All of the identified variants were synonymous. One of the synonymous variants, of which the Dachshund case was heterozygous for, was predicted by Ensembl VEP to affect splice regions [15]. This variant was present in the DBVDC at an allele frequency of 0.04. While the overall effect of this variant was considered undetermined, its heterozygous state in the affected dog along with the presence of a more compelling causal variant made it unlikely the cause of xanthinuria in the Dachshund. The other 9 synonymous variants were considered likely neutral and not the cause of disease.

3.4. Population analysis

3.4.1. Manchester and English Toy Terriers

The allele frequency of the putative causal *MOCOS* c.232G > T (p.Gly78Cys) variant in a population of 386 Manchester Terriers was 0.13. Variant genotype frequencies are summarized in Table 4. Eighty-eight dogs were heterozygous for the variant and 3 were homozygous. The three homozygous dogs were a 3-month-old male and two 4-year-old female littermates with no history of xanthine urolithiasis. A urine sample was obtained from the male puppy, and infrared spectroscopy analysis identified xanthine crystals; insufficient urine was available for further analysis. Urine was also obtained from the two female Manchester Terriers, and xanthine was quantified by liquid chromatography. The results showed 20–57 fold higher intensity of xanthine in the urine of the *MOCOS* c.232G > T homozygotes compared to a healthy mixed-breed female dog that was clear of all the xanthinuria-associated variants (Fig. 3).

Variant frequency was also determined in a population of 285 English Toy Terriers (Table 4), a breed that is closely related to, and interbred with, Toy Manchester Terriers. The allele frequency of the *MOCOS* c.232G > T variant in this population was 0.10; no English Toy Terriers were homozygous for the variant.

During the study, we also received a DNA sample from a Manchester Terrier with historic urolithiasis that tested homozygous for the *MOCOS* c.232G > T variant. The dog was a seven-week-old intact male that was a littermate to one of the Manchester Terriers with xanthine uroliths and close relative of the other (Fig. 4). He had a history of uroliths analyzed at a non-veterinary laboratory and reported to be composed of urate; the stones were not available for re-analysis.

3.4.2. Manchester Terrier pedigree analysis

Pedigrees were available for most of the Manchester Terriers included in the study. Pedigrees were analyzed to determine relationships between all dogs homozygous for the *MOCOS* c.232G > T variant, including the two Manchester Terrier dogs with confirmed xanthine uroliths, the suspected case (reported as urate), and the three subclinical xanthinuria cases. The pedigree was consistent with an autosomal recessive mode of inheritance. A potential founding father, 2–3 generations back from all homozygotes and within 5 generations of most carriers, was identified (Fig. 4). He was heterozygous for the *MOCOS* c.232G > T variant; however, his parents were not available for testing.

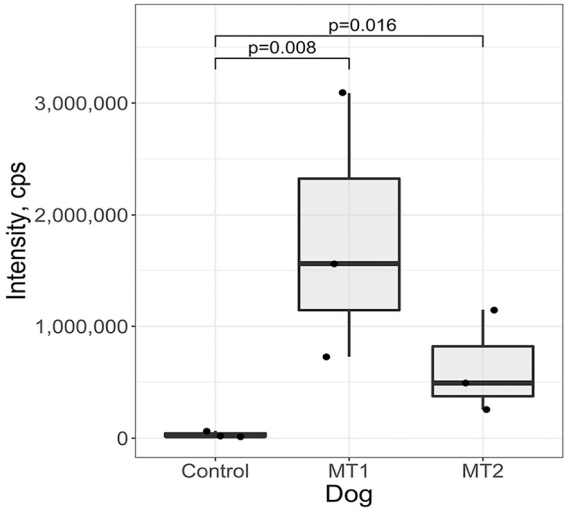

Fig. 3. Urine xanthine intensity from a healthy female dog, clear of putative causal xanthinuria variants (control) and 2 MT females homozygous for the putative causal *MOCOS* c.232G > T variant (MT1 and MT2). Box and whisker plot representing interquartile range of the data; the horizontal line represents the median and the whisker bars extend to 1.5× the interquartile range. Dots represent the technical replicates for each dog. Urine xanthine levels were significantly ($p < 0.05$) higher in the two homozygous females than the control dog.

Table 4
Genotype frequency of the putative causal variants in *MOCOS* and *XDH* in cases with xanthine uroliths, breed population controls, and the Dog Biomedical Variant Database Consortium (DBVDC).

	XDH c.654G > A		
Genotypes	**T/T**	**T/C**	**C/C**
Mixed Breed Case	0	0	1
DBVDC	813	0	0
Total	813	0	1
	***MOCOS* c.232G > T**		
Genotypes	**G/G**	**G/T**	**T/T**
Manchester Terrier Cases	0	0	2
Manchester Terrier Controls	295	88	3
English Toy Terrier Controls	227	58	0
DBVDC	813	0	0
Total	1337	146	5
	***MOCOS* c.383delC**		
Genotypes	**C/C**	**C/del**	**del/del**
CKCS cases	0	0	3
CKCS controls	105	3	1
English Cocker Spaniel cases	0	0	1
English Cocker Spaniel controls	42	0	0
DBVDC	813	0	0
Total	960	3	5
	***MOCOS* c.137 T > C**		
Genotypes	**T/T**	**T/C**	**C/C**
Dachshund cases	0	0	1
Dachshund controls	116	0	0
DBVDC	813	0	0
Total	929	0	1

CKCS, Cavalier King Charles Spaniel.

3.4.3. Cavalier King Charles Spaniels and English Cocker Spaniels

The allele frequency of the *MOCOS* c.383delC variant was 0.03 in a population of 109 Cavalier King Charles Spaniels. Variant genotype frequencies are summarized in Table 4. Three dogs were heterozygous for the variant, and 1 dog was homozygous for the variant. Medical records were not available for the homozygous dog. The *MOCOS* c.383delC variant was not found in a population of 42 English Cocker Spaniels.

During the study, we also received a DNA sample from a fourth Cavalier King Charles Spaniel with urolithiasis that tested homozygous for the *MOCOS* c.383delC variant. The sample was submitted for genetic testing due to clinical suspicion for hereditary xanthinuria, but the dog was not included in the main case cohort as the uroliths were not analyzed (they had been shipped to the MUC for analysis but lost during transit). The dog was a 16 month old, spayed female, evaluated for nephroliths, obstructive ureteroliths, and cystoliths diagnosed with ultrasonography and computed tomography. The cystoliths and ureteroliths were surgically removed and described as having a green/grey color, as is typical for a purine composition. The dog had no evidence of a portosystemic vascular anomaly on abdominal imaging and no history of therapy with an XDH inhibitor.

3.4.4. Dachshunds

The *MOCOS* c.137 T > C variant was absent from a population of 116 Dachshunds.

3.4.5. Mixed-breed

The breeds of origin for the mixed breed dog were unknown. Therefore, no specific breed population was tested for the *XDH* c.654G > A variant. However, the variant was not present in the DBVDC

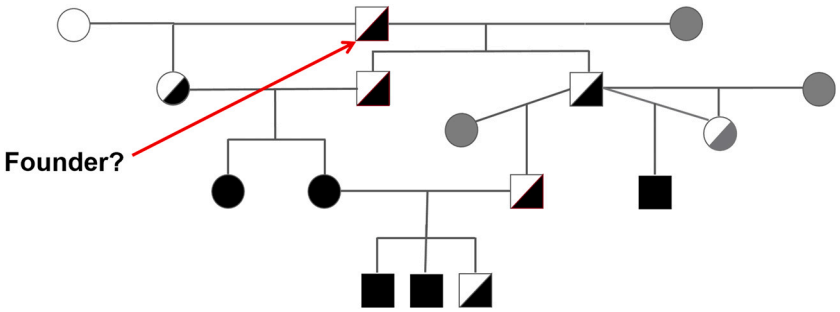

Fig. 4. Condensed pedigree showing the identification of a potential founding father. The potential founding father for the *MOCOS* c.232G > T putative xanthinuria variant was located 2–3 generations back from the cases and within 5 generations of most of the carriers in the tested breed population. Full shading = T/T, half shading = G/T, no shading = G/G. Black and white = known genotypes, grey = genotype unknown.

(Table 4).

4. Discussion

In this study, we identified putative causal variants in *XDH* and *MOCOS* in four dog breeds and a mixed-breed dog with a history of xanthine uroliths. All variants were found in a homozygous state in the affected dogs, consistent with an autosomal recessive mode of inheritance. This demonstrates that *XDH* and *MOCOS* are shared susceptibility genes in dogs and humans. The variants discovered in this study varied in molecular subtype and gene and provide insight into potential critical domains in both *XDH* and *MOCOS*.

One of the variants identified in this study was located in *XDH* and thus this condition was classified as Xanthinuria Type I. This synonymous SNP in the last base pair *XDH* exon 8 (c.654G > A), detected in a mixed-breed case, resulted in an in-frame splicing error. Analysis of cDNA revealed the substitution results in exon skipping, removing 30 amino acids from the translated sequence. Because this is an in-frame variant, it is unknown whether the aberrant splicing results in XDH deficiency due to insufficient transcript levels for activity or if the enzyme is present but functioning incorrectly. PhyloP scores revealed the removed residues are highly conserved, and InterProScan localized them to the FAD-binding domain (Fig. 1). The removed residues have not been previously reported to be crucial for enzyme function [2]. However, the presence of disease associated with their absence infers an important functionality. It is also possible that the removal of exon 8 removes portions of the protein which are important for forming or maintaining protein structure. Although functional analyses to determine the importance of these amino acids was not within the scope of this study, our data suggests that exon 8, encompassing residues 189–218 (ENSCAFT00000047701.2), is critical for *XDH* function.

Three of the four variants identified in this study were located in *MOCOS* and classified as causing Xanthinuria Type II. In the Manchester Terriers, we identified a missense variant in *MOCOS* (c.232G > T; p.Gly78Cys), which results in an in-frame splicing error. Similar to the mixed-breed case, cDNA analysis revealed that this variant results in exon skipping and removes 30 amino acids. As stated for the Xanthinuria Type I variant, we do not know if alternative splicing results in disease due to inadequate transcript expression or protein dysfunction. PhyloP scores showed that the removed residues were highly conserved over this region, and InterProScan localized them to the aminotransferase class V domain (Fig. 2). The association of the variant with xanthinuria supports suggests key functionality of these amino acids. Again, an alternative possibility is that the residues lost are important for protein structure. The results of this study suggest that residues 48–77 of exon 2 (ENSCAFT00000028243.4) are critical for *MOCOS* function.

Another Xanthinuria Type II variant was identified in a Dachshund case and occurred in *MOCOS* exon 1 (c. 137 T > C; p.Leu46Pro). As with the exon-skipping variants, we did not determine whether this missense variant exerts its effect through directly disturbing enzyme function or via an effect on protein structure. However, MutPred2 predicted effects of the variant on protein structure, including gain of an intrinsic disorder and loss of helix, as well as altered ordered interface [17]. Multiple missense variants have previously been reported humans and other species with Xanthinuria Type II, including exon 1 variants, but none are located in close proximity (within 50 amino acids) of the dog variant [22–24].

The final putative Xanthinuria Type II variant identified in this study is a 1 bp deletion (c.383delC; p.Ala128Glyfs*30) in exon 4 of *MOCOS* present in both Cavalier King Charles Spaniel and English Cocker Spaniel cases. The deletion causes a frameshift, resulting in the formation of a premature stop codon 89 bp downstream. While we did not investigate *MOCOS* activity, it is unlikely that the truncated protein would function due to the loss of more than 80% of the normally expressed protein, including three important domains (Fig. 2). The truncated mRNA transcript is likely eliminated by nonsense mediated decay. Nonsense and frameshift variants have been previously reported to cause Xanthinuria Type II in humans and other species [3,23,25–27].

Ideally, the molecular subtypes for each xanthinuria case would be confirmed with metabolic testing. Measurement of metabolites in the urine, such as N1-methyl-2-pyridone-5-carboxamide and N1-methyl-4-pyridone-5-carboxamide, can be used to distinguish type 1 from type 2 xanthinuria. A MOCOS deficiency affects not only XDH but also aldehyde oxidase activity. N1-methyl-2-pyridone-5-carboxamide and N1-methyl-4-pyridone-5-carboxamide are the product of N1-methylnicotinamide oxidation by aldehyde oxidase. Thus, their excretion is normal in the case of type 1 xanthinuria but reduced in the case of type 2 xanthinuria [28,29]. Urine samples for the cases of each xanthinuria type were unavailable for these metabolic analyses, limiting the classification of types to presumptive based on the gene harboring the putative causal variant.

The *MOCOS* putative causal variant (c.232G > T) identified in Manchester Terriers had the highest allele frequency in the respective breed populations at 0.14. A single, common, male ancestor was identified 2–3 generations back from the cases and is believed to be the founding father (Fig. 4). He was determined to be heterozygous for the variant. However, his parents were not available for testing to determine if the variant was inherited or occurred de novo in him. We suspect that this variant quickly reached a high frequency in the breed due to a popular sire effect [30]. Another similar breed, developed in England, is the English Toy Terrier; pedigrees show interbreeding between Manchester Terriers and English Toy Terriers and in our breed population. The English Toy Terriers screened in this study had a *MOCOS* c.232G > T variant frequency of 0.07.

Although Cavalier King Charles Spaniels and Dachshunds are the most represented breeds in the previous xanthine urolithiasis literature, the allele frequencies of the xanthinuria variants (*MOCOS* c.383delC and c.137 T > C) were low in the respective breed populations at 0.03 and 0. English Cocker Spaniels share a putative causal variant for xanthinuria with Cavalier King Charles Spaniels, supporting that this variant arose

before the breeds diverged. Xanthinuria reports in Cavalier King Charles Spaniels indicated that elevated prevalence might occur in the United Kingdom [6–9]. Thus, the low variant frequency in our North American cohort might not be representative of populations in other continents. Reports of hereditary xanthinuria in Dachshunds originated in European countries (Germany, France, and Czech Republic) [10–12]. Again, the absence of the putative causal variant in our Dachshund breed population might not reflect variant frequency outside the United States.

A limitation of this study is that the breed populations, and thus the variant frequencies, could be affected by selection bias. In the case of the Manchester Terriers, most samples for the breed population screening were submitted for genetic testing, and breeders with dogs related to affected or known carriers might be more likely to test. The other breed populations largely originated from internal and external biobanks, which also might not reflect the general breed population.

We identified three Manchester Terriers homozygous for the *MOCOS* c.232G > T variant that did not have a history of urolithiasis. All three were subsequently diagnosed with presumed or confirmed xanthinuria using infrared spectroscopy and liquid chromatography, respectively. None of these dogs were screened with medical imaging and might have also had subclinical uroliths. In humans, up to two-thirds of individuals with hereditary xanthinuria are asymptomatic with clinical signs only manifesting if urolithiasis develops [31–33]. Urolith formation can be influenced by other biologic and environmental factors such as sex and diet, as well as urine properties such as osmolality, pH, and concentrations of pro- and anti-calculogenic substances [34]. In addition, one Manchester Terrier homozygous for the *MOCOS* c.232G > T had a history of urate, not xanthine uroliths. However, the uroliths were analyzed by a non-veterinary laboratory and were not available for re-analysis. It is possible for purine stones to be misanalysed as urate; one example being 2,8-dihydroxyadenine uroliths [35].

Animal models offer the opportunity to discover critical protein domains, determine pathologic consequences associated with molecular subtypes of disease, and serve in pre-clinical testing of novel therapies. The dog is a naturally occurring animal model for both type I and type II hereditary xanthinuria [6–12]. Other naturally occurring models include cat [36–39], cow [26,27,40], sheep [41], and goat [24]. A naturally occurring model could be applied to investigate the correlation between genotype and phenotypic severity. At this time, there is no treatment for xanthinuria other than a low purine diet and increased water consumption [1]. Natural animal models could also benefit development and testing of potential treatments.

5. Conclusion

We identified 4 putative causal variants in 4 dog breeds and a mixed breed dog affected with xanthine urolithiasis. Although functional analyses were not within the scope of this study, the results suggest the affected amino acids might have a critical role in enzyme function. Dogs with naturally occurring hereditary xanthinuria could be utilized in further studies to develop and test potential treatments.

Funding

Partial support for EF was provided by a National Institute of Health ORIP K01 Mentored Research Scientist Development Award (K01-OD019912). Partial support was also provided by generous donations from Manchester Terrier owners and breeders. The funders had no role in the study design, collection, analysis, and interpretation of data, decision to publish, or preparation of the manuscript.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgements

The authors would like to acknowledge the Minnesota Urolith Center technicians for their assistance with obtaining data on xanthine submissions and spectroscopy of the urine samples, the study breeders and owners who participated in the study, for submitting samples, sharing their pedigrees and knowledge, and the veterinarians, Drs. Tim Kuhnmuench, Michael Koch, Mary Beth Callan, and Kate Byrne, for sharing medical histories and submitting DNA from cases.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2021.100792.

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