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STANDARD ARTICLE

Novel genetic variant associated with globoid cell leukodystrophy in a family of mixed breed dogs

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Abstract

Background: Globoid cell leukodystrophy (GCL) is a fatal autosomal recessive disease caused by variants in the *galactosylceramidase* (*GALC*) gene. Two dog breed-specific variants are reported.

Objectives: Characterize the putatively causative *GALC* variant for GCL in a family of dogs and determine population allele frequency.

Animals: Four related mixed-breed puppies with signs of neurologic disease were evaluated. Subsequently, 33 related dogs were tested for genetic markers for parentage and the identified *GALC* variant. Additional *GALC* genotyping was performed on 278 banked samples from various breeds.

Methods: The 4 affected puppies had neurological exams and necropsies. DNA was isolated from blood samples. Variants in *GALC* were identified via Sanger sequencing. Parentage testing was performed using short tandem repeat markers. Prevalence of the *GALC* variant of interest was investigated in other breeds.

Results: GCL was confirmed histopathologically. A novel missense variant in *GALC* (NC_006590.4:g.58893972G>A) was homozygous in all affected animals (n = 4). A recessive mode of inheritance was confirmed by parentage testing as was variant linkage with the phenotype (LOD = 3.36). Among the related dogs (n = 33), 3 dogs were homozygous and 7 heterozygous. The variant allele was not detected in screening 278 dogs from 5 breeds. The novel variant is either unique to this family or has an extremely low allele frequency in the general population.

Conclusions and Clinical Importance: A novel *GALC* variant was identified that likely explains GCL in this cohort. The identification of multiple causal variants for GCL in dogs is consistent with findings in humans.

KEYWORDS
canine, congenital disease, *GALC*, Krabbe disease, neurologic disease

Abbreviations: DLA, dog leukocyte antigen; *GALC*, galactosylceramidase gene; GCL, globoid cell leukodystrophy; H&E, hematoxylin and eosin; PCA, principal component analysis; PCR, polymerase chain reaction; STR, short tandem repeat; VGL, Veterinary Genetics Laboratory.

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1 | INTRODUCTION

Globoid cell leukodystrophy (GCL), also known as Krabbe disease, is a congenital autosomal recessive inherited disease that affects humans, dogs, and many other species.[1] Clinical onset in dogs is usually between 1 and 3 months of age and signs include progressive limb paresis, tremors, and muscular atrophy.[2] Death occurs by 1 year of age, though dogs are typically humanely euthanized before then. At the tissue level, there is failure of degradation during normal homeostatic turnover of galactocerebroside, the primary lipid component of the myelin sheath.[3] The lack of degradation of this lipid results in the accumulation of psychosine, a toxic metabolite. The specific mechanism by which psychosine accumulation results in clinical signs is not fully elucidated, though a current theory is that it acts by disrupting membrane architecture.[4] Diagnosis of GCL includes observation of clinical signs as well as histopathological staining of the neurologic tissue after death to observe the characteristic globoid cells.[5]

Causative variants for GCL have been localized to the *galactosylceramidase* (*GALC*) gene. In humans, over 100 different *GALC* variants have been found to result in Krabbe disease.[6,7] In dogs, 2 causal mutations have been reported. The first variant, c.473A>C, resulting in a tyrosine to serine substitution with consequent low *GALC* activity, was discovered in West Highland White and Cairn Terriers.[2] The second variant was reported in Irish Setters and is a complex in-frame 78 bp insertion (c.790_791insAF260905.1) that results in a longer than normal, but inactive protein.[8] Although clinical reports of GCL have appeared in multiple other breeds, there has been no evaluation of whether they were also caused by the previously reported variants.[5,9] Given the reported abundance of *GALC* variants underlying GCL in humans, it is not unreasonable to posit that canine variants may be breed-specific or even private to families.

The purpose of this study was to elucidate the specific putative causative *GALC* variant in a family of mixed breed dogs with histopathologically confirmed GCL, to verify the mode of inheritance of this mutation, and to measure the allele frequency of this variant in a broader population of dogs.

2 | MATERIALS AND METHODS

2.1 | Animals

Three 4-month-old related mixed-breed puppies presented to the University of Illinois Urbana-Champaign Veterinary Teaching Hospital for clinical evaluation of progressive signs of neurologic disease. A complete neurological exam was performed. Based on presumptive Border Collie heritage, blood samples had been submitted by the referring veterinarian for measurement of cobalamin and for genetic testing for Imerslund-Gräsbeck syndrome. Based on progression of clinical signs and poor prognosis, the 3 puppies were humanely euthanized and underwent complete necropsy including banking of whole blood and histopathology. One year later, a fourth related puppy presented for euthanasia and necropsy evaluation after developing the same clinical signs at 5 months of age. An additional 33 related dogs were voluntarily surrendered to an animal shelter and underwent mandated spay/neuter procedures including banking of whole blood. Three of these dogs subsequently developed signs of neurologic disease, including hind limb ataxia, but were not presented for further diagnostic work-up. DNA samples from unrelated dogs used during this study were collected during an unrelated project (IACUC protocol #21035) or were part of the DNA bank at the University of California-Davis Veterinary Genetics Laboratory (VGL).

2.2 | Pathology/Histology

Post-mortem examination was performed on all 4 clinically affected puppies. The brain and spinal cord were removed from each animal and placed in 10% neutral buffered formalin at a 1:10 tissue: formalin volume ratio for at least 48 hours. Formalin-fixed tissues were then trimmed into cassettes and routinely processed, paraffin-embedded, and sectioned at 4 μm onto glass slides followed by automated hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS) staining.

2.3 | DNA isolation

DNA was isolated from whole blood samples from the 37 putatively related dogs using a commercial kit (Qiagen PureGene DNA isolation kit, Qiagen, Germantown, Maryland, USA) following manufacturer protocols and subsequently frozen at −20°C. Other DNA samples used in this study were from those banked at the VGL (278 samples across 5 breeds) and by 1 of the investigators (AMM; 1 each Golden Retriever, Australian Cattle Dog, Galgo, Border Collie, and Boston Terrier).

2.4 | Parentage testing and breed analysis

As the specific relationships among the 37 putatively related dogs were unknown, parentage testing was performed to establish relationships and mode of inheritance for the affected dogs. The breed of these dogs was suspected to be Border Collie based on phenotype, but this could not be confirmed by the owner. Therefore, principal component analysis (PCA) of pairwise genetic distance was also performed to determine likely breed(s). DNA from these dogs were genotyped using markers from the commercially available Parentage and Diversity Panels at the VGL (https://vgl.ucdavis.edu/test/parentage-genetic-marker-report-dog and https://vgl.ucdavis.edu/test/canine-genetic-diversity). All 37 dogs were tested for 65 short tandem repeat markers (STRs). Genotypes from 48 STRs were used to test possible trio combinations for mating exclusions. A trio combination was considered if the sire and dam were at least 6 months older than the potential offspring or if the age was unknown. Parents qualified if there were 0 or 1 exclusion for the tested trio. In cases where a trio had 2 or more exclusions, parents were evaluated separately. Genotypes from 33 STR markers were used in a PCA of pairwise genetic distance. Dog leukocyte antigen (DLA) haplotypes (DLAI

and DLA II) were determined based on genotypes from 7 STRs in this region. Two dogs failed to produce genotypes for all 40 markers used for PCA and DLA haplotype analysis; therefore, 35 of the study cohort dogs were compared to VGL data from 1079 dogs from 55 breed or breed groups to determine potential breed(s) assignment (Table S1).

2.5 | PCR and sequencing for GALC variants

Primers for *GALC* were designed using Primer3 software[10,11] using the CanFam3.1 assembly.[12] Primers for the *GALC* investigation are detailed in Table S2. PCR protocol was as follows: 95°C for 20 minutes; then 35 cycles of 95°C for 30 seconds, 55°C or 57°C (depending on primer pair, see Table S2) for 30 seconds, 72°C for 1 minute; then 72°C for 15 minutes; then hold at 4°C. DNA fragments were visualized on 2% agarose gels with ethidium bromide to verify fragment size and quality. Sequencing products were purified (DNA Clean and Concentrator-5 kit, Zymo Research, Irvine, California, USA) before Sanger sequencing at the University of Illinois Roy J. Carver Biotechnology Center. Sequences were aligned to CanFam3.1 and traces were manually screened for variants utilizing commercial software (Sequencher DNA sequence analysis software version 5.4.6, Gene Codes Corporation, Ann Arbor, Michigan, USA). Predicted pathogenicity of variants were determined by the consensus classifier PredictSNP,[13] PANTHER,[14] MutPred2,[15] and PolyPhen-2.[16] Discovery sequencing of the complete coding sequence and untranslated regions (UTRs) of *GALC* was performed in 3 dogs, including 1 confirmed affected puppy, 1 putative parent, and 1 unrelated individual. Subsequently, genotyping by sequencing was performed in the entire study cohort (37 related and 5 unrelated dogs). For the purpose of this report, variant descriptions have been updated to reflect the most recent CanFam6 assembly, following HUGO nomenclature guidelines (https://varnomen.hgvs.org/). Linkage testing for the identified variant of interest with disease status was performed based on the established pedigree using LAMP software (version 0.0.9),[17,18] applying the "—recessive" disease model for calculating maximum likelihood (LOD) score.

2.6 | Allele frequency testing

To investigate if the identified variant of interest was private to this cohort or found in other breeds, 278 dogs from 5 breeds (Collie n = 45, Doberman n = 46, Italian Greyhound n = 45, Scotch Collie n = 45, Border Collie n = 97) were also genotyped for the *GALC* variant by MassARRAY technology (Agena Bioscience, Inc., San Diego, California, USA). Breeds were selected based on the breed assignment analysis. Primers were designed using the MassARRAY Typer Assay Design software v5.0.1 (5′ Capture Primer: ACGTTGGATGTGTTGTGTGGTGTGC ATCAG, 3′ Capture Primer: ACGTTGGATGTTGAGAACGATAGGG CTCTG, Extend Primer: AGAAGTCGGGAGGTT). Samples were amplified using iPLEX Gold Reagents, and dogs with genotypes confirmed by Sanger sequencing were used as positive controls for each possible genotype (G/G, G/A, A/A). Data were analyzed using the TyperAnalyzer v5.0.2 (Agena Bioscience, Inc., San Diego, California, USA).

3 | RESULTS

3.1 | Clinical/Neurological findings

The primary presenting complaint for the 3 clinically affected puppies presented for diagnostic examination was difficulty walking, incontinence, and head tremors. All had a similar history of pelvic limb ataxia and paraparesis that progressed to paraplegia and the development of urinary and fecal incontinence over the course of approximately 8 to 10 weeks. The puppies all had cobalamin levels within the acceptable reference range and tested negative for Imerslund-Gräsbeck syndrome. Before presentation, the puppies had been treated with doxycycline and clindamycin, and 1 puppy received a course of prednisone. No improvement was seen with treatment.

Three puppies were presented for examination by the Neurology service (the fourth puppy presented post-euthanasia for necropsy). There were no abnormalities noted on general physical examination. A neurologic examination was performed by a board-certified neurologist (DWH). All 3 puppies had a normal mentation and cranial nerve examination. The motor function of the 3 puppies varied. One puppy was paraplegic with absent nocioception in the pelvic limbs, 1 puppy was paraplegic with intact nocioception in the pelvic limbs, and 1 puppy was nonambulatory paraparetic. All puppies had a short-strided gait in the forelimbs when supported, absent postural reactions in the hind limbs, decreased patellar reflexes and withdrawals, and marked muscular atrophy in the hind limbs and lumbar epaxial muscles. All puppies were urinary incontinent and leaked urine when supported to walk. All 3 puppies displayed marked muscle atrophy and increased muscle tone in the hind limbs.

All 3 puppies showed a mixture of peripheral and central nervous system signs. Given the increased muscle tone, slightly diminished reflexes, and lack of ataxia with nonambulatory paraparesis/plegia, a motor neuron localization was highly prioritized. The suspected diagnosis was a congenital neurodegenerative disease process. Based on the severity of clinical signs and their progression, humane euthanasia was elected for all the affected animals.

3.2 | Gross and histopathological findings

Among the 3 puppies that presented at the same time, 2 had muscle atrophy in the pelvic limbs and presumptive cerebellar atrophy in the brain. The least severely affected puppy only showed regional muscle atrophy in the hind limbs. When their 3 brains were compared grossly, the most severely affected puppy had a smaller brain with flattened cerebral gyri and a thinner brainstem (Figure 1). The fourth puppy, which presented a year later, had similar regional muscle atrophy in the hind limbs with urine scalding. With no comparison present, the brain of the fourth puppy did not have any gross abnormalities.

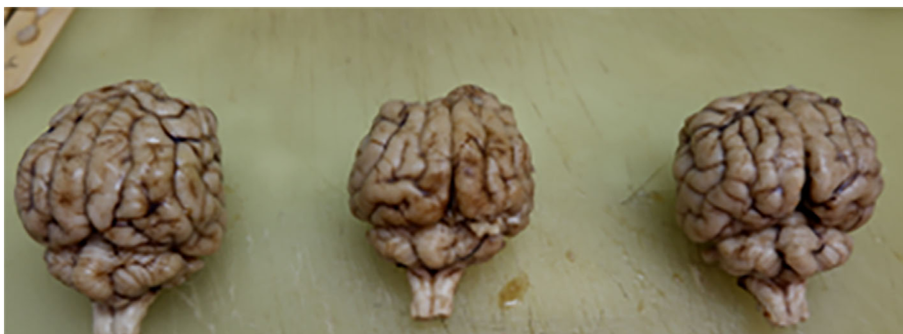

FIGURE 1 Gross appearance of brains from the 3 affected dogs examined at the same time. The middle brain was from the most severely clinically affected dog. It is grossly smaller than the other 2 and has flattened cerebral gyri and a thinner brainstem.

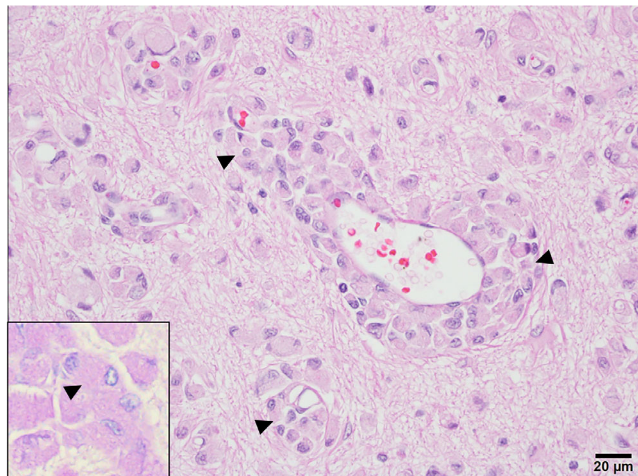

FIGURE 2 Photomicrograph of cerebral cortical white matter with plump globoid macrophages (arrowheads) filled by Periodic acid-Schiff-positive material (inset) infiltrating perivascular spaces and scattered in the neuropil of 1 of the affected dogs. H&E stain.

On histopathological examination of the cerebrum and cerebellum as well as along the length of the spinal cord, there were mild-to-moderate numbers of plump macrophages multifocally following white matter tracts, obscuring the junction between gray and white matter, and infiltrating perivascular spaces (Figure 2). These macrophages contained moderate to abundant amounts of amphophilic, fibrillar to flocculent cytoplasm that occasionally peripheralized and compressed the nucleus. These macrophages were occasionally binucleated and identified as globoid cells with positive cytoplasmic PAS staining. The macrophages also occasionally infiltrated into the adjacent white matter parenchyma which was variably expanded by edema. Diffusely, there was mildly increased cellularity within the gray matter. Based on the presence of the characteristic globoid cells, it was concluded that all 4 of the puppies had GCL.

3.3 | Sequencing and genotyping

The complete coding sequence and UTRs of *GALC* were sequenced in 3 dogs (including 1 confirmed affected puppy) for the purpose of variant detection. Neither of the mutations previously reported to be causative for GCL[2,8] were present in these dogs (determined via Sanger sequencing). Instead, a novel mutation in Exon 2 of *GALC* was discovered (NC_006590.4:g.58893972G>A). This missense variant resulted in an alanine to valine residue substitution (NP_001003238.1:p.(Ala50Val)) and was predicted to be deleterious to protein function by PredictSNP with 76% confidence. Similar predictions were made by the other classification algorithms (Table S4). The variant was confirmed by sequencing to be homozygous in each of the 4 known affected dogs. Ultimately, Exon 2 was sequenced in all 37 putatively related dogs to genotype the NC_006590.4: g.58893972G>A variant. In total, 7 dogs were homozygous (including all 4 with confirmed GCL) and 7 heterozygous for this variant. The variant was not present in any of the 5 unrelated dogs genotyped by sequencing. Linkage between genotype at the novel mutation and disease status (LOD 3.36, $P < .0001$) supported the conclusion that this mutation is causative for disease in this family.

3.4 | Parentage testing

Thirty-five out of the 37 putatively related dogs in the study cohort could be placed within likely family trees (Figure 3). Both sire and dam were assigned for 20 dogs; 18 trios had 0/48 mating exclusions, 1 trio had 0/47, and 1 trio had 1/48. One dog (#49) had 2 possible dams (#29 and #30) with 0/48 exclusions for both trios. This dog was placed in the pedigree as the offspring of dam #30 because of the close age of all dogs that qualify as offspring of sire #28 and dam #30. The structure of these familial relationships supported the expected autosomal recessive mode of inheritance of the variant (Figure 3). Parentage analysis suggested that a single carrier male (#28) sired all puppies with 2 copies of the variant (n = 7) and 4 of the 6 identified carriers. One other identified carrier (#57), when mated to (#28), produced 4 of the homozygous puppies. One additional mate of (#28) is suspected to be a carrier as she (denoted as unknown) produced 3 of the histopathologically confirmed affected dogs homozygous for the variant. Based on parentage testing performed, this unknown dam was not sampled as part of this study. Dogs #26 (unaffected) and #64 (unaffected) could not be placed in the pedigree because 2 sires qualified (#28 and #62) with 0/48 exclusions and no mating qualifications with less than 3 exclusions.

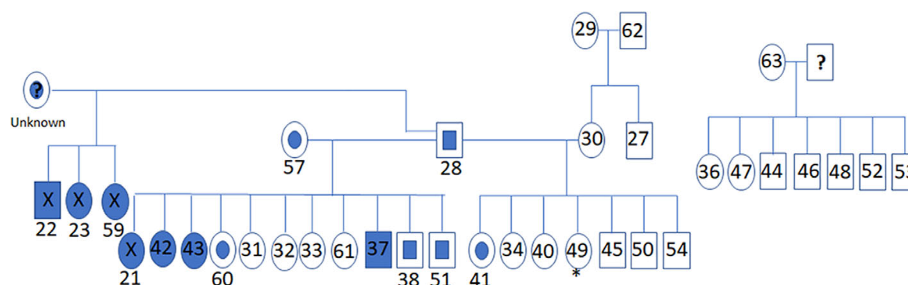

FIGURE 3 Proposed relationships between dogs included in the study cohort based on parentage analysis. Two unaffected individuals could not be placed within this family organization. Dogs #21-23 were from litters born in late 2017; the remainder of the offspring from these same matings were born in early 2019. Solid fill = homozygous for novel variant and showing clinical signs of disease; dogs labeled with an X are those confirmed affected with globoid cell leukodystrophy based on histopathology. Partially filled = heterozygous for novel variant (carriers). Open = homozygous for reference allele. Dogs labeled with a question mark (?) have proposed genotypes based on mode of inheritance but their identity is unknown. * Two dams qualify with 0/48 exclusions for the trio (#30 and #29). The sample was placed on this pedigree as offspring of #30 because potential littermates are the same age.

3.5 | Breed analysis and allele frequency testing

PCA of STR data supported the supposition that the study cohort was not comprised of pure-bred dogs. When compared to 55 diverse breeds, these data suggest the study cohort dogs are crossbred Border Collies (Figure S1 shows the relationships of the 5 most closely related breeds to the study cohort). Based on DLA haplotypes, there was additional support for Border Collie as the DLA haplotype denoted as 1160-2098, which is a fairly common haplotype in the Border Collies (14.75%), was detected in 10 dogs in the study cohort. The only other breed identified in the VGL database, comprising 16 068 entries, with this haplotype is the Saint Bernard (1.15% frequency). Additionally, the DLA haplotype denoted as 1040-2039 has only been identified in Italian Greyhound (7.87%) and Doberman Pinchers (0.91%) and 21 dogs in this sample set had this haplotype providing evidence to support Italian Greyhound ancestry with Doberman also a possibility. Based on the PCA analysis, dogs of 5 breeds with potential shared ancestry with banked samples at the VGL were investigated for the novel *GALC* variant. However, the novel *GALC* variant identified in the study cohort was not found in any of the 278 dogs tested (Table S3).

4 | DISCUSSION

Here, we document a familial occurrence of GCL in 4 mixed-breed dogs and characterize a novel *GALC* missense variant resulting in a predicted protein conformation change that is likely causative for the disease in this study cohort. This is the third *GALC* variant reported in dogs that has been associated with GCL in various breeds, but the first in a family of presumptive Collie ancestry. Testing for this novel mutation in additional dogs from breeds with possible shared ancestry did not reveal any dogs with the novel allele. This suggests that the variant is either unique to this cohort or that the allele frequency in the larger canine population is rare and would require testing many dogs from different breeds to determine a true population allele frequency. Given the nature of the study cohort, it is possible that the mutation arose de novo in a parent of the heterozygous sire and dams and has been propagated through close inbreeding.

Limitations of this study include the relatively small sample size, including missing individuals from the putative family tree. Clinical assessment of the homozygous and heterozygous individuals who were not a part of the proband group was not available, so clinical disease in any of these dogs or their offspring cannot be confirmed. However, 3 additional puppies from the litters of the 4 confirmed affected individuals did have signs of neurologic disease reported by the referring veterinarian in the months following the diagnosis of their littermates; this is consistent with the number of homozygote dogs (n = 7) in this family. While protein predictions support that this missense variant is deleterious, functional protein assays were not performed, therefore, the specific mechanistic effects of the NC_006590.4: g.58893972G>A variant remain unknown.

Thousands of different variants have been found for all of the varying lysosomal storage diseases in humans,[19] making the discovery of an apparently private variant not unexpected. There are currently no antemortem tests for GCL in the canine model, though diagnostic biomarkers are being investigated.[20] As supportive care, which can only slow progression of clinical signs, is the only treatment option for GCL in any species,[21] elucidation of causal genetic variants is an important component of disease prevention as it allows breeders to avoid at-risk matings. While the development of a commercial assay for the variant reported here is not warranted at this time given that it appears to be localized to this family or is exceedingly rare, this work highlights the opportunity for targeted sequencing and genetic testing in pedigrees in which a genetic disease is suspected, but existing reported variants are not present. As genomic technologies become increasingly affordable, this type of precision medicine should become more widely available to veterinary clinicians in practice.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Samples used in this study were either provided by the owner or referring veterinarian for diagnostic purposes and banking (IACUC exempt) or collected under IACUC protocol #21035 (University of Illinois Urbana-Champaign).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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