



## Neuronal ceroid lipofuscinosis in a German Shorthaired Pointer associated with a previously reported *CLN8* nonsense variant



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### ARTICLE INFO

#### Keywords:

Lysosomal storage disease

Dog

Neurodegeneration

Whole genome sequence

### ABSTRACT

Two littermate German Shorthaired Pointers, a male and a female, were adopted as puppies from an animal shelter. Both puppies developed normally until approximately 11 months of age when the male began to exhibit neurological signs including ataxia, vision loss, and behavioral changes indicative of cognitive decline. These signs increased in severity over time. The female remained neurologically normal and healthy. The affected dog was euthanized at approximately 21 months of age. Autofluorescent cytoplasmic storage bodies were detected in neurons in unstained tissue sections from the cerebellum, the cerebrum, and the retina. Electron micrographs of these storage bodies showed that they were membrane bound and that most contained tightly packed aggregates of membranous whorls along with a variety of other ultrastructural features. This ultrastructure, along with the autofluorescence and the clinical signs supported a diagnosis of neuronal ceroid lipofuscinosis (NCL). Unlike earlier investigated forms of canine NCL with causal alleles in *ATP13A2*, *TPP1*, *MFSD8* and *CLN5* that had autofluorescent cytoplasmic storage bodies in cardiac muscle, no autofluorescence was detected in cardiac muscle from the affected German Shorthaired Pointer. A 39-fold average coverage whole genome sequence indicated that the affected German Shorthaired Pointer was homozygous for the A allele of a G > A transversion at position 30,895,648 chromosome 37. This 37:30895648G > A mutation created a *CLN8* termination codon that had been previously reported to cause NCL in a mixed breed dog with Australian Shepherd and Australian Cattle Dog ancestry. This nonsense allele was heterozygous in the clinically normal female sibling, while archived DNA samples from 512 other German Shorthaired Pointers were all homozygous for the reference allele. The affected German Shorthaired Pointer and the previously diagnosed mixed breed dog with the same nonsense mutation shared an identical homozygous haplotype that extended for 4.41 Mb at the telomeric end of chromosome 37, indicating the both dogs inherited the nonsense mutation from a common ancestor.

### 1. Introduction

Lysosomal storage diseases associated with pathogenic alleles in at least 13 genes have been classified as neuronal ceroid lipofuscinoses (NCLs) based on similarities in clinical and pathological disease phenotypes [1]. In affected human subjects, apparently normal early development is followed by progressive neurological decline characterized by signs that include impaired motor and cognitive functions, loss of vision, seizures, and impairments in respiration and swallowing. Most forms of NCL culminate in premature death. The age of onset and rate of disease progression vary depending on the gene involved and the specific causal allele within that gene. A characteristic feature of the

NCLs is massive accumulation of autofluorescent lysosomal storage bodies in the central nervous system and other organs and tissues [2]. The clinical signs are usually accompanied by progressive brain atrophy and retinal degeneration. The NCLs are classified as forms CLN1 through CLN14 based on the gene in which the pathologic sequence variant occurs [1]. Clinical subtypes within many of the different CLN forms have been described [3,4].

Naturally occurring NCLs resulting from causal alleles in the canine orthologs of 8 of the CLN genes have been identified in purebred and mixed breed dogs [5,6]. The onset of clinical signs in the canine NCLs ranges from a few months to 7 years of age depending on the responsible gene and the causal allele it harbors [5]. In one dog breed a

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<https://doi.org/10.1016/j.ymgmr.2019.100521>

Received 13 August 2019; Accepted 11 September 2019

Available online 21 October 2019

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complex sequence variant in the gene associated with the CLN1 form of NCL was associated with progressive retinal atrophy without any of the other signs that are typical of this disease [7]. Nonsyndromic sequence variants associated retinal pathology alone have been reported for *CLN3* and *CLN7* (*MFSDB8*) in human subjects [8–10].

We have identified the majority of the sequence variants that underlie the forms of canine NCL that have been described to date [5]. The process by which these discoveries have been made starts when a dog owner or veterinarian reports to us a dog that is exhibiting behavioral signs suggestive of NCL. We request veterinary medical histories from the referring veterinarians and information about behavioral abnormalities from the dogs' owners. We also request blood samples for DNA isolation and pedigree information, if available. When the dogs are euthanized, we request that brains, retinas, and other tissues be collected and that samples from each type of tissue be fixed in separate fixatives for both light and electron microscopic examinations. If a tentative diagnosis of NCL is supported by the presence of storage bodies that autofluoresce in unstained tissue and exhibit characteristic ultrastructural features in brain and retina, we either screen for known canine NCL-associated sequence variants or generate whole genome sequences to identify the causal sequence variants. In this study we performed these analyses for NCL identification using samples from an unregistered German Shorthaired Pointer that was euthanized after exhibiting progressive neurological signs that are typical of canine NCL. To our knowledge, NCL has not been reported previously in this dog breed.

## 2. Materials and methods

### 2.1. Subject dogs

These studies were conducted with the informed consent of the dogs' owner and were approved by the University of Missouri Institutional Animal Care and Use Committee. Two littermate German Shorthaired Pointer puppies, one male and the other female, were adopted as pets by the same person from an animal shelter. The dogs were spayed or neutered prior to adoption. Both dogs had the typical appearance of purebred German Shorthaired Pointers (Fig. 1).

At approximately 11 months of age the male began to exhibit a loss of coordination that grew progressively worse over time. The dog became clumsy, ataxic, and bumped into stationary objects, walls, and people in a familiar environment. By 15 months of age he exhibited compulsive circling and tongue movements, jaw chattering, and was confused by familiar sounds. From that time forward there was of a progressive decline in the ability to understand and respond to

previously learned commands and other behavioral cues and the dog began to regress in previously learned housetraining. At 17 months of age the dog exhibited obvious visual impairment in both bright and dim light, and by 18 months the dog was hesitant to attempt to climb up or down stairs. No seizure activity or trance-like behavior was observed by the dog's owner and the dog never become aggressive. The dog became progressively anxious between 18 and 21 months of age and was videotaped during this period. Video recordings illustrating the affected dog's abnormal behaviours are included in the supplemental material. At approximately 21 months of age, the dog was humanely euthanized due to the progression of behavioral signs. The affected dog's female littermate has exhibited no significant behavioral abnormalities at over two years of age.

### 2.2. Clinical evaluations and necropsy

The affected dog was examined by a veterinary ophthalmologist at approximately 18 months of age. The dog exhibited bilateral absences of visual tracking and menace responses. Direct and indirect pupillary light reflexes and dazzle reflexes were present when stimulated by a bright light source. No abnormalities were noted by fundic examinations. The dog was diagnosed with central blindness with undetermined cause and was referred for a neurological examination. At 18 months age the dog had obtunded mentation and severe proprioceptive ataxia in all limbs. No other neurological abnormalities were observed. No significant abnormalities were detected with standard complete blood count and blood chemistry analyses. Upon re-examination at 21 months of age, no direct or indirect pupillary light reflexes could be elicited.

The dog was humanely euthanized at 21 months of age following the final neurological examination. Immediately after euthanasia, the eyes were enucleated and the corneas were removed. One eye was immersed in a fixative solution consisting of 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4. The other eye was immersed in a fixative consisting of 3.5% paraformaldehyde, 0.05% glutaraldehyde, 0.12 M sodium cacodylate, pH 7.4 (Immuno fix). The entire brain was then removed and bisected longitudinally. One half was frozen for potential future biochemical analyses. From the other half of the brain, one slice each of the parietal cerebral cortex and of the cerebellum were immersed in Immuno fix. Adjacent slices from each region were immersed in a fixative consisting of 2.0% glutaraldehyde, 1.12% paraformaldehyde, 0.13 M sodium cacodylate, pH 7.4 (EM fix). The remainder of this half of the brain was immersed in 10% buffered formalin (Fisher Chemical, Cat. no. SF93–4). Slices of the heart ventricular wall were obtained and preserved with the same fixatives as were used for brain tissue preservation. Each tissue slice was



Fig. 1. Photographs of the affected male dog a few weeks prior to euthanasia at 21 months of age (left) and of his unaffected female littermate at approximately two years of age.

approximately 3 mm thick. All fixed tissues were maintained at ambient temperature until processed for microscopic examination. All samples were shipped from the neurologist in Florida to the University of Missouri via express overnight delivery.

### 2.3. Microscopic analyses

The eyes were dissected and areas from the central posterior region of each eye adjacent to the optic nerve head were prepared and examined with fluorescence, light and electron microscopy as described previously [11]. Portions of the brain regions, retina and heart ventricle that were preserved in Immuno fix were embedded in paraffin and unstained sections of the paraffin blocks were examined with fluorescence microscopy [12].

Small areas of the central posterior retina fixed in 2.5% glutaraldehyde and of the cerebral cortex and cerebellar cortex preserved in EM fix were processed and sectioned for electron microscopic examination [13,14]. The samples were washed in cacodylate buffer, post-fixed with osmium tetroxide, and embedded in epoxy resin. Sections of each sample were cut to thicknesses of 70 to 90 nm and were mounted on thin-bar copper grids. The sections were then stained with uranyl acetate and lead citrate and were subsequently examined and imaged using a JEOL JEM-1400 transmission electron microscope equipped with a Gatan digital camera.

### 2.4. DNA analysis

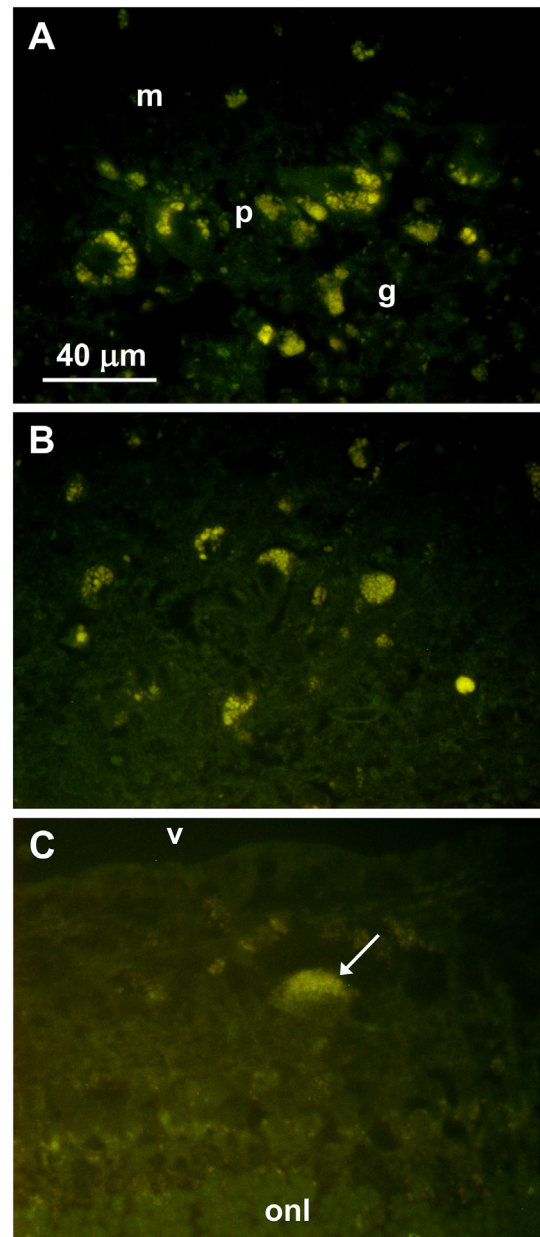
The canine DNA samples used in this study were purified from EDTA anti-coagulated blood as previously described [15]. DNA from the affected dog was submitted to the McDonnell Genome Institute for PCR-free library preparation and sequencing on the Illumina NovaSeq platform. A previously described modified best-practices GATK pipeline [16] was used for alignment of the resulting paired-end sequence data to the reference genome (CanFam3.1) and for calling variants. Ensembl annotation (version 89) and commercial software (Golden Helix, SVS version 8.8.3) were used for producing variant reports. Rare variants were identified by comparison to 146 other whole genome sequences of dogs with a variety of diseases including 6 dogs with suspected or confirmed NCL. Prior to whole genome sequencing, a previously described TaqMan allelic discrimination assay [14] was used to genotype the affected dog for the 22:30,574,637C > T transition in *CLN5* responsible for NCL in a variety of breeds. A different TaqMan allelic discrimination assay [17], for a G-to-A transition at 37:30883950 in *CLN8*, was used to genotype the sibling of the affected dog and archived DNA samples from German Shorthaired Pointers.

## 3. Results

### 3.1. Histopathology and electron microscopy

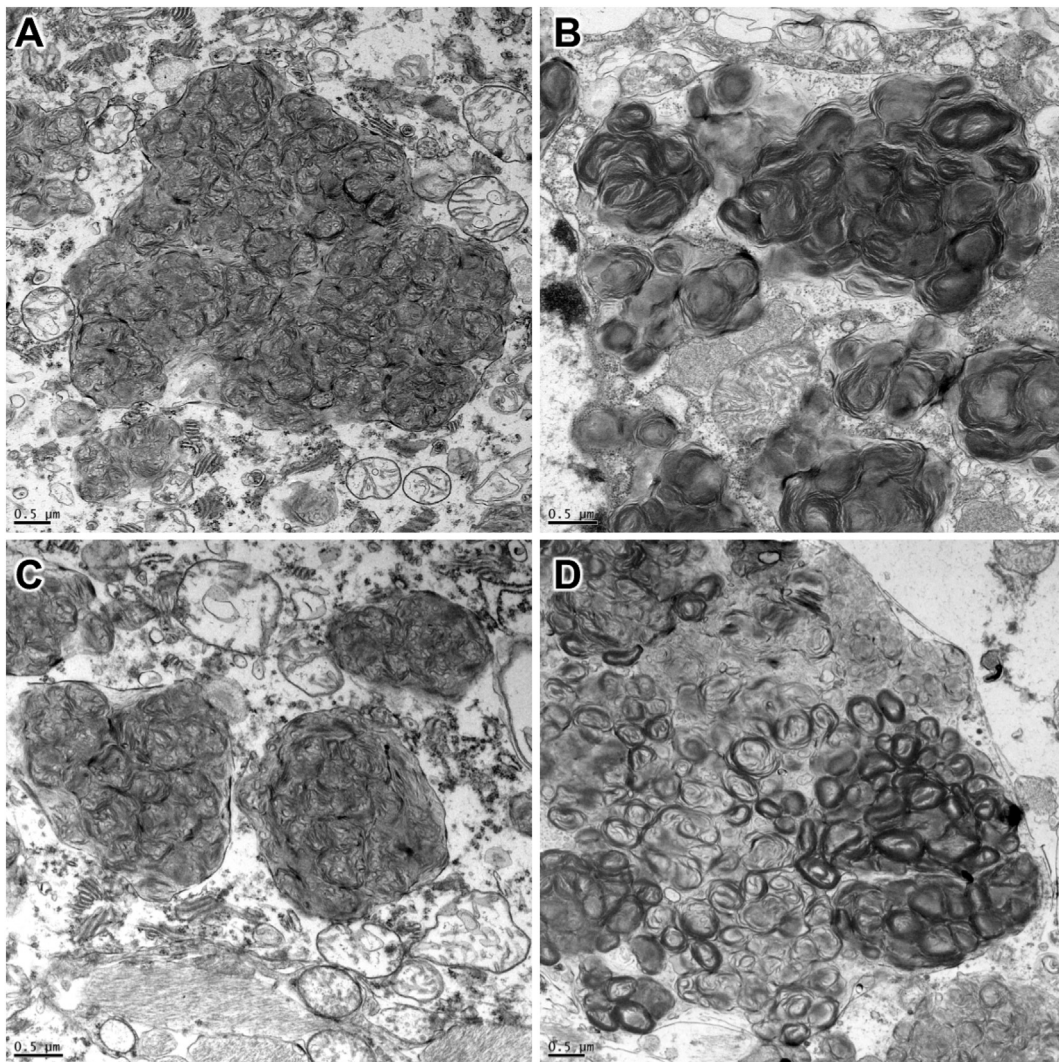
Brain, retina and heart samples from the affected dog were examined for the presence of autofluorescent lysosomal storage material that is characteristic of the NCLs. Substantial amounts of autofluorescent storage material were present in cerebellar Purkinje cells as well as in other cells within the Purkinje cell layer (Fig. 2A). In addition, cells with large amounts of the autofluorescent material were present in the granule cell layer, with fewer cells containing this material in the molecular layer (Fig. 2A). Cells with substantial accumulations of disease-specific autofluorescent granules were abundant throughout the cerebral cortex and appeared to occur in the majority of cortical neurons (Fig. 2B). In the retina, accumulation of these autofluorescent granules was restricted primarily to the ganglion cells (Fig. 2C). The cardiac muscle was devoid of the autofluorescent storage material observed in other forms of canine NCL.

The ultrastructural appearances of the disease-specific storage bodies in the cerebellum of the affected dog were similar in the Purkinje



**Fig. 2.** Fluorescence micrographs of the cerebellar cortex (A), the parietal lobe of the cerebral cortex (B), and the inner retina (C). In the cerebellum, the greatest accumulation of the disease-specific autofluorescent material was in the Purkinje cell layer (p), both within Purkinje cells and between them. Neurons throughout the cerebellar cortex contained substantial amounts of the autofluorescent material, primarily in the cell bodies adjacent to the nuclei (B). In the retina, aggregates of the autofluorescent granules were present in the ganglion cell bodies adjacent to the nuclei (arrow in C). No disease-specific autofluorescence was observed in the ganglion cell axons, in the Muller cell end-feet adjacent to the vitreous, or in cells of the inner and outer nuclear layers.

cells (Fig. 3A and B) and in cells in the granule cell layer (Fig. 3C and D). There was some variability in the shapes and substructures of the storage bodies, even within the same cell type (compare Fig. 3A and B). The storage bodies were membrane-bounded and their contents consisted primarily of tightly packed aggregates of membranous whorls and vesicles. The diameters of most storage body profiles ranged from 1.5 to 6 μm. However, in some cells in the granule cell layer, a large proportion of the entire cell body was occupied by a single large storage body that could be as large as 20 μm in diameter (Fig. 3D).



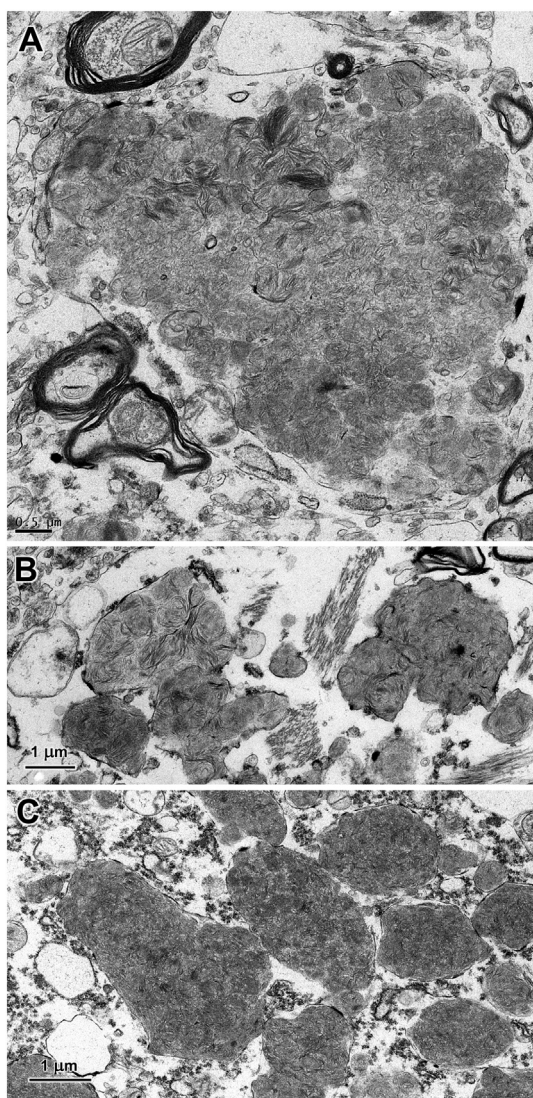
**Fig. 3.** Electron micrographs of storage bodies in cerebellar Purkinje cells (A and B) and in cells in the molecular cell layer (C and D) of the affected dog. All of the storage bodies were filled with whorls of membrane-like structures. One very large storage body occupied almost the entire cell body of a cell in the molecular cell layer (D). Storage bodies of this large size were not observed in the Purkinje cells.

As with the cerebellum, the storage body contents in the cerebral cortex and the retinal ganglion cells consisted primarily of aggregates of membrane-like structures (Figs. 4 and 5). However, in these tissues there was much more variability in the appearance and organization of the storage bodies (Figs. 4 and 5). In the cerebral cortex, the sizes and appearances of the storage bodies varied greatly between cells, but within individual cells, each of the storage bodies were similar in appearance (Fig. 4C). The sizes and appearances of the storage bodies within the retinal ganglion cells was even more variable. Stacks of membrane-like structures arranged in parallel arrays were only found within the storage bodies of the retinal ganglion cells (arrow in Fig. 5), although these comprised only a fraction of the material within the storage bodies of this cell type.

### 3.2. Molecular genetic analyses

Because a *CLN5* nonsense mutation at 22:30,574,637 has been found to be responsible for NCL in a variety of breeds [18], we first genotyped the affected German Shorthaired Pointer for this *CLN5* variant and determined that the dog was homozygous for the reference allele. To identify the molecular genetic cause for the NCL in the current case, we used DNA from the affected dog to generate a whole genome sequence with 39.4-fold average coverage. Golden Helix SVS software

and Ensembl annotation were used to produce a variant report with data from the whole genome sequence of the affected dog and also from the whole genome sequences of 146 other dogs with a variety of diseases that served as controls. Relative to the reference sequence, the whole genome sequence of the affected dog contained 20,638 variant alleles predicted to alter the amino acid sequence of the encoded polypeptides. Only 20 of these variant alleles, harbored by 18 different genes, were homozygous in the affected German Shorthaired Pointer and absent from the other 146 whole genome sequence in our data set (Supplementary Material, Table 1). None of these 20 alleles have been previously associated with NCL-like disorders and, based on their known biological functions, none were considered candidates for causality. We therefore relaxed our selection criteria to find variant alleles that were homozygous in the affected dog and rare, but not absent, in the control whole genome sequences. The variant allele of a G-to-A transition at 37:30883950 was homozygous in the affected German Shorthaired Pointer and also homozygous in the whole genome sequence of a mixed breed dog, but absent from the other 145 whole genome sequences in our reference data set. The mixed breed dog was previously reported by us to have NCL caused by the homozygous 37:30883950A allele which predicts a nonsense mutation in *CLN8* (p.Trp195\*) [17]. The clinically normal sibling of the affected German Shorthaired Pointer was an A/G heterozygote at 37:30883950.



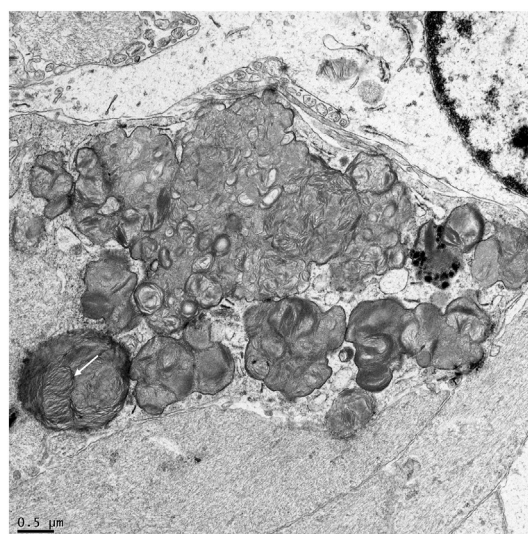
**Fig. 4.** Electron micrographs of disease-specific storage bodies in neurons of the parietal cerebral cortex from the affected dog. The contents of the storage bodies in all neurons consisted primarily of membrane-like material, but the organization of the membrane-like profiles varied between storage bodies and even within a single storage body. Images A, B, and C were each taken from a different cell.

Archived DNA samples from 512 German Shorthaired Pointers all tested homozygous for the 37:30883950G allele.

To determine whether or not the 37:30883950A alleles in the affected German Shorthaired Pointer and in the affected mixed breed dog stemmed from the same founding event, we generated a Golden Helix SVS variant report that contained all chromosome 37 variants that occurred in either or both of the canine whole genome sequences of these dogs. These two dogs were homozygous for an identical haplotype that started at position 37:26491131 extended for 4.4 Mb to the telomeric end of chromosome 37. This indicates that both dogs inherited their *CLN8* nonsense mutation from a common ancestor.

#### 4. Discussion

To date 13 sequence variants have been associated with different forms of NCL in dogs (Table 1). In almost all cases, the variants have been restricted to a single dog breed or to two similar breeds. The one exception has been a *c.619C > T* transition in *CLN5* that converts a glutamine codon to a termination codon (p.Gln207Ter). This variant



**Fig. 5.** Electron micrograph of disease-specific storage bodies in a retinal ganglion cell from the affected dog. As in other tissues, the contents of the storage bodies were membrane-like in appearance. In some of the storage bodies the membrane-like structures were arranged as stacks of parallel profiles (arrow).

was first reported in Border Collies [19] and was subsequently also found in the similar Australian Cattle Dog breed [14]. At the time of the latter discovery, it was assumed that the existence of the same variant in both breeds was due to cross breeding between Border Collies and Australian Cattle dogs, both of which are often kept by the same or neighboring owners who use them for herding. More recently this *CLN5* variant was found to be homozygous in a mixed breed dog with no apparent Border Collie or Australian Cattle Dog ancestry [18]. Haplotype analysis indicated that there was a single founding mutation event which may have predated the establishment of the modern dog breed registries. Thus, the *CLN5* disease allele may be segregating in as yet unidentified breeds and was considered a candidate for the cause of the disorder in the affected German Shorthaired Pointer. After the known *CLN5* NCL variant was ruled out, whole genome sequence analysis was elected as the most efficient means of identifying the disease variant in the affected dog.

Four different *CLN8* variants have been reported to cause canine NCL (Table 1). Previously, three of these variants had each been associated with a single breed: English Setter, Alpenländische Dachsbracke, and Saluki. This breed-restricted distribution of causal alleles would be expected if the founding mutations occurred in members of already established breeds. The other causal *CLN8* variant was found in a cross-breed dog that resulted from the mating of an Australian Shepherd with an Australian Cattle Dog. Because members of both of the parental breeds are similar herding dogs, interbreed matings are likely to be common. Thus, it appeared that the homozygous *CLN8* variant responsible for the NCL in this mixed breed dog could have originated in a member of either of the parental breeds. We here report that the identical *CLN8* variant in the homozygous state was responsible for NCL in a member of a very different breed, the German Shorthaired Pointer.

As expected, the German Shorthaired Pointer and the mixed breed dog with the same homozygous causal variant in *CLN8* exhibited a very similar patterns of clinical signs [17]. One exception to this was that the mixed-breed dog exhibited generalized seizures near end-stage disease when 21 months of age. Generalized seizures were not observed in the affected German Shorthaired Pointer who was euthanized at the same age. Since the seizures started in the mixed-breed dog just prior to euthanasia, it possible that seizures would have occurred in the affected German Shorthaired Pointer had the dog been allowed to survive longer.

**Table 1**  
Summary of canine NCL-associated disease sequence variants.<sup>a</sup>

Disease	Gene	Sequence variant	Amino acid change	Affected dog breed
CLN1	<i>PPT1</i>	c.736_737insC	p.F246Lfs*29	Dachshund [20]
CLN1	<i>PPT1</i>	c.124 + 1G > A	Splice variant	Cane Corso [12]
CLN2	<i>TPP1</i>	c.325delC	p.A108Pfs*6	Dachshund [21]
CLN5	<i>CLN5</i>	c.619C > T	p.Q207X	Border Collie [19] Australian Cattle Dog [14] Mixed breed [5,18]
CLN5	<i>CLN5</i>	c.934_935delAG	p.E312Vfs*6	Golden Retriever [22]
CLN6	<i>CLN6</i>	c.829 T > C	p.W277R	Australian Shepherd [23]
CLN7	<i>MFSD8</i>	c.491 T > C	p.F282Lfs*13	Chinese Crested [13] Chihuahua [24,25]
CLN8	<i>CLN8</i>	c.491 T > C	p.L164P	English Setter [15]
CLN8	<i>CLN8</i>	c.585G > A	p.W195X	Australian Shepherd & Australian Cattle Dog [17] German Shorthaired Pointer <sup>b</sup>
CLN8	<i>CLN8</i>	g.30852988_30902901del	CLN8 absence	Alpenländische Dachsbracke [26]
CLN8	<i>CLN8</i>	c.349dupT	p.Glu117*	Saluki [6]
CLN10	<i>CTSD</i>	c.597G > A	p.M199I	American Bulldog [27]
CLN12	<i>ATP13A2</i>	c.1623delG	p.P541 fs*56	Tibetan Terrier [28]
CLN12	<i>ATP13A2</i>	c.1118C > T	p.Thr373Ile	Australian Cattle Dog [29]

<sup>a</sup> Updated from previous published list [5].

<sup>b</sup> This report.

The contents of the storage bodies in all cell types examined in both 37:30883950A homozygous dogs consisted primarily of similar aggregates of membrane-like material. Thus, breed background does not appear to have a noticeable effect on storage-body ultrastructure. Although the clinical signs of the NCLs are primarily neurological, the association of these diseases with impaired lysosomal function suggests that disease pathology is likely to occur in extra-neuronal tissues because almost all cell types have lysosomes. Since our observation that progressive cardiac pathology occurs in Dachshunds with the CLN2 form of NCL [30], we have been examining the cardiac muscles obtained at necropsy from dogs suspected of suffering from NCL. We have found substantial accumulations of autofluorescent storage material in the cardiac muscles from dogs suffering from NCL due to disease alleles in *ATP13A2* [29], *TPP1* (*CLN2*) [12], *MFSD8* [25], and *CLN5* [14]. Whether this is the case for other forms of NCL remains to be determined. The German Shorthaired Pointer evaluated in this study gave us our first opportunity to examine heart muscle from a *CLN8*-deficient dog for autofluorescent storage body accumulation. Somewhat surprisingly, no disease-related autofluorescence was detected in the cardiac ventricular muscle of this dog. This suggests that the pattern of extra-neuronal tissues and organs that exhibit autofluorescence varies according to the gene harbouring the disease-causing sequence variant.

The causal homozygous *CLN8* nonsense alleles in the affected German Shorthaired Pointer and in the previously reported mixed-breed dog with Australian Shepherd and Australian Cattle Dog ancestry are imbedded in a shared homozygous haplotype that extends for over 4.4 Mb at the telomeric end of chromosome 37. This indicates that the causal alleles in both affected dogs stem from a common ancestor and that there was a single founding mutation event. The nonsense allele appears to be rare in all three of the above mentioned breeds. Archived DNA samples from 512 German Shorthaired Pointers all tested homozygous for the reference allele. As reported earlier, 144 Australian Cattle Dogs all tested homozygous for the reference allele and only 3 of 1484 NCL-free Australian Shepherds were heterozygotes (nonsense allele frequency equals 0.001) [17]. Thus, for most purposes, screening healthy dogs of these breeds or other breeds for the nonsense allele would not be cost effective. Nonetheless, in the future we plan to screen canine NCL suspects from any breed background for the *CLN8* 37:30883950A allele and also for the *CLN5* 22:30,574,637 T allele before we invest in whole genome sequencing to identify novel causes for canine NCL. We can provide these tests without charge to investigators, veterinarians, and dog owners with access to dogs with an NCL-like clinical histories of unknown cause.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmgmr.2019.100521>.

## Acknowledgements

This work was funded in part by grant EY023968 from the U.S. National Institutes of Health and Grant No. 02604 from AKC Canine Health Foundation, United States.

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