

## A mutation in the cathepsin D gene (*CTSD*) in American Bulldogs with neuronal ceroid lipofuscinosis

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### Abstract

We obtained DNA, brains, and eyes from American Bulldogs with neurodegeneration due to neuronal ceroid lipofuscinosis (NCL). The diagnosis of NCL was confirmed by detection of autofluorescent cytoplasmic inclusions within neurons throughout the brains, in retinal ganglion cells, and along outer limiting membranes of the retinas. Electron microscopy revealed that the inclusions had coarsely granular matrices surrounding well-delineated spherical structures and that the inclusions near the retinal outer limiting membranes were within photoreceptor cells, mostly cones. Affected American Bulldogs were homozygous for the A allele of a G to A transition in the cathepsin D gene (*CTSD*), which predicts the conversion of methionine-199 to an isoleucine. Only the G allele was detected in DNA samples from 131 randomly selected dogs representing 108 breeds other than American Bulldog; however, the A allele had a frequency of 0.28 among 123 genotyped American Bulldogs. Transmission analysis in a 99 dog pedigree of American Bulldogs indicated a probability of less than  $10^{-7}$  that alleles from any mutation unlinked to *CTSD* would be concordant with the pedigree and phenotypes of the dogs. Brain samples from affected dogs had 36% of the cathepsin D-specific enzymatic activity found in control dog brains; whereas, specific enzymatic activities of 15 other lysosomal enzymes were unchanged or increased. Compared to previously described NCLs in mice and sheep that completely lack cathepsin D activity, the clinical course of NCL in the American Bulldogs was less severe and more closely resembled that of many human NCLs.

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**Keywords:** Neuronal ceroid lipofuscinosis; *CTSD*; Cathepsin D; Canine; Missense mutation

### Introduction

The neuronal ceroid lipofuscinoses (NCLs) are a group of heritable diseases characterized by progressive neuronal degeneration and the accumulation of autofluorescent cytoplasmic inclusions in the brain, retina, and other tissues. NCLs have been described in people, mice, and a vari-

ety of domestic mammals including sheep and dogs. Human NCLs have been attributed to mutations in six genes: *PPT1*, *CLN2*, *CLN3*, *CLN5*, *CLN6*, and *CLN8* [1–7]. Mutations in three additional genes are associated with NCL in animals. Sheep homozygous for a missense mutation in the cathepsin D gene (*CTSD*) are born with severe NCL [8]. Furthermore, knockout mice rendered nullizygous for *CTSD*, *CLCN3* or *PPT2* develop forms of NCL [9–11]. Canine NCLs have been diagnosed in at least 18 breeds where they are important both as veterinary diseases and as

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potential models for human NCLs ([http://www.caninegeneticdiseases.net/CL\\_site/formsCL.htm](http://www.caninegeneticdiseases.net/CL_site/formsCL.htm)). Previous studies have found a *CLN8* missense mutation associated with NCL in English Setters [12] and a *CLN5* nonsense mutation associated with NCL in Border Collies [13].

A young-adult onset NCL occurs in American Bulldogs [14]. Affected dogs typically exhibit hypermetria and ataxia before two years of age. Psychomotor deterioration progresses slowly but relentlessly, leading to death before seven years of age. Cytoplasmic autofluorescent storage material is present within neurons in the brains and retinal ganglion cells of affected dogs [14]. We obtained DNA from affected individuals and began sequencing exons from the candidate genes mentioned above. We here report evidence that a missense mutation in *CTSD* is responsible for NCL in American Bulldogs.

## Materials and methods

One hundred and twenty-three EDTA-anticoagulated blood samples were obtained from privately owned American Bulldogs and DNA was extracted as previously described [12]. The owners of these dogs provided pedigree information and submitted a form detailing their animal's relevant clinical history ([http://www.caninegeneticdiseases.net/CL\\_site/formsCL.htm](http://www.caninegeneticdiseases.net/CL_site/formsCL.htm)). Three of the American Bulldogs were available for necropsy as described below. Another 131 canine DNA control samples representing 108 breeds other than American Bulldog were randomly selected from the University of Missouri Canine DNA Repository.

To confirm the clinical diagnoses and further characterize American Bulldog NCL, the brains and eyes of three terminally affected American Bulldogs were collected at necropsy, immediately after the dogs were euthanized. Brain samples from these dogs were promptly frozen and stored at  $-70^{\circ}\text{C}$  as were similarly collected brain samples from four age-matched normal control dogs. The eyes and portions of the cerebral cortex and cerebellum were preserved for fluorescence microscopy (FM), and electron microscopy (EM). Cryostat sections obtained from the FM samples were examined for storage body autofluorescence as previously described [15]. Ultrathin tissue sections were examined and photographed with a JEOL 1200 EX transmission electron microscope.

All 9 *CTSD* exons were amplified by PCR with primers designed from sequences in GenBank contig numbers AAEX01015066.1 and AAEX01015067.1 which contain the gene. Annealing temperatures and oligonucleotide sequences for each primer pair are listed in Table 1. Because of the high G/C content in and around exon 1, 1 M betaine was included in the amplification reaction mixture for this exon. Amplicons were sequenced in both directions as previously described [16]. Pyrosequencing with a PSQ 96 instrument was used for genotyping the DNA samples with respect to a missense mutation in the *CTSD* gene. The PCR primers were 5'-biotinyl-TTCATCGCGCCAAGTT-3' with 5'-TGTC

GAAGACAGGCAGCAC-3'; and, the sequencing primer was 5'-GATG CGGGGGTAGGC-3'. Enzyme-specific activities in cerebral cortex tissues from three American Bulldogs with terminal NCL and from four age-matched control dogs were determined as previously described [8].

We constructed an extended pedigree of 56 genotyped dogs aged 2 years and greater for which phenotype was known without error (8 affected and 48 normal) and 43 non-genotyped relatives, which provided pedigree relationships among the genotyped animals. We then derived the probability that a randomly sampled biallelic mutation would be concordant with the pedigree and phenotypes as a function of the unknown minor allele frequency  $q$ . For this calculation, all obligate carriers were uniquely assigned heterozygous genotypes, all affected animals were assigned homozygous genotypes for the minor allele (assumed to be the disease-associated allele), and all other animals with normal phenotypes were allowed to be either heterozygous or homozygous for the major allele. Genotype probabilities were computed for each animal within the pedigree based upon transmission probabilities and the assumption that the minor allele was the recessive disease-causing mutation. The probability that a randomly sampled biallelic mutation was concordant with the pedigree and phenotypes is:  $P_C = \sum_S \prod_i P_S(P_i|G_i)P_S(G_i)$ , where  $P_S(G_i)$  is the probability of the genotype of the  $i$ th individual within the pedigree,  $P_S(P_i|G_i)$  is the probability of the phenotype of the  $i$ th individual given the individual's genotype and the summation is across all possible sets ( $S$ ) of genotypes that are concordant with the pedigree and phenotypes. Since, we assume that the genotypes are fully penetrant,  $P_S(P_i|G_i) = 1$  for all genotypes that are concordant with the observed phenotype. Consequently,  $P_C$  reduces to  $\sum_S \prod_i P_S(G_i)$ . This probability was maximized ( $P_C = 10^{-13.64}$ ) at  $q = 0.27$  which is consistent with the value of 0.28 obtained from the total sample of 123 genotyped individuals. From this, we estimate the probability that there is no other mutation within the dog genome that is concordant with the pedigree and phenotypes as  $P_0 = (1 - P_C)^N$ , where  $N$  is the number of independent biallelic mutations within the dog genome. Thus, the probability that there are any other mutations within the dog genome that are consistent with the pedigree and phenotypes is  $1 - P_0$ .

## Results

All three of the affected American Bulldogs available for necropsy had massive accumulations of autofluorescent storage bodies in the cerebellum, cerebral cortex, and retina. In addition to the substantial accumulations of storage material in the retinal ganglion cells, autofluorescent inclusions were observed along the outer limiting membrane of the retina adjacent to the outermost layer of photoreceptor nuclei (Fig. 1). At the ultrastructural level, the storage bodies were observed to be membrane-bound cellular inclusions with cross-sectional diameters generally ranging from 0.5 to 3  $\mu\text{m}$  (Fig. 2). The inclusions had coarsely granular matrices. Often embedded within the matrices were

Table 1  
Primers and conditions for amplification of canine *CTSD* exons

| Exon           | Forward primer/reverse primer 5'-3' sequence | Annealing temp ( $^{\circ}\text{C}$ ) | Amplicon size (bp) |
|----------------|--|---------------------------------------|--------------------|
| 1 <sup>a</sup> | CCGGCTATAAGCGCACGACCT/CGTGGGACGCACACCGGGTCT  | 68                                    | 366                |
| 2              | GGGAGCCCTGATGAACCCT/GCCCATACAGAACCTTCCCTAA   | 58                                    | 292                |
| 3              | AGCCTTAATGGACCCTGCTC/CCATAGTCTGCCTGCCTCCT    | 56                                    | 203                |
| 4              | GCTGGCTGAGACCAAGGC/AGCCGTCTGAGCATCCC         | 58                                    | 298                |
| 5              | TTGAGTCTGGGTGCGTGGCCCTCT/AAAGCTGCCGCTCGTCTG  | 63                                    | 437                |
| 6              | GTGGCTGACTCCGCTTCT/CCTGCTCCCAAGGGCTGTA       | 58                                    | 260                |
| 7              | CCTGAAGGAGGGGACTGC/CCTCTGAGGACAGCTTGTAAGT    | 58                                    | 393                |
| 8              | GTGGACGAGGTGCGTGAGCT/CGCCTTGCGAAACCTG        | 58                                    | 331                |
| 9              | CCTGCTGGACTCCTTCCCT/TGCTTACCACCCTGGCTAT      | 53                                    | 227                |

<sup>a</sup> Amplification mixture for exon 1 contained 1 M betaine.

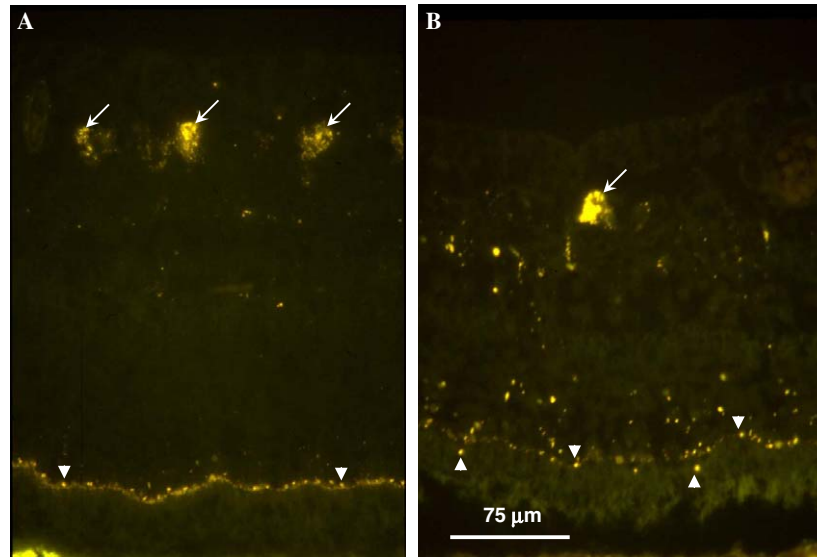


Fig. 1. Fluorescence micrographs of central (A) and peripheral (B) regions of the retina from an American Bulldog that was euthanized after exhibiting clinical signs of NCL. In the central retina, autofluorescent storage bodies were present primarily in the ganglion cells (arrows in A) and along the inner side of the outer limiting membrane (arrowheads in A). In the peripheral retina, storage bodies were present not only in the ganglion cells (arrow in B) and along the inner side of the outer limiting membrane (downward pointing arrowheads in B), but also on the photoreceptor inner segment side of the outer limiting membrane (upward pointing arrowheads in B). Bar in (B) indicates magnification for both micrographs.

well-delineated spherical inclusions that were either more darkly or more lightly stained than the surrounding matrix. With EM it could be seen that the storage bodies near the outer limiting membrane were in photoreceptor cells, with the majority of the inclusions occurring within cone cells (Fig. 2B). The ultrastructural appearances of the storage bodies in the photoreceptor cells were similar to those previously reported in other neural cell types in the affected American Bulldogs [14].

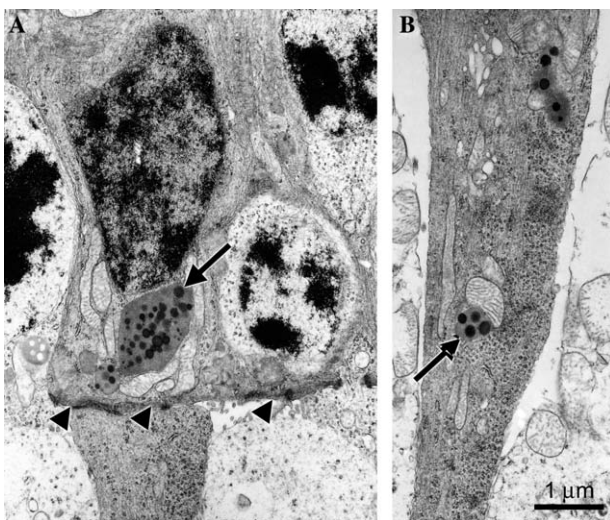


Fig. 2. Electron micrographs demonstrating the presence of disease-specific storage bodies in photoreceptor cells. Storage bodies (arrow in A) were found within photoreceptor cells just interior to the outer limiting membrane (arrowheads in A), as well as less frequently in the photoreceptor inner segments (arrow in B).

Nucleotide sequences were determined for all nine *CTSD* exons from an American Bulldog confirmed with FM and EM analyses to have NCL. These sequences were compared to corresponding sequences in GenBank contigs AAEX01015066.1 and AAEX01015067.1 from the Boxer DNA sequence produced in the canine genome project (<http://www.genome.gov/12511476>). The American Bulldog sequences from exons 1, 2, 3, 4, 6, 7, 8, and 9 were identical to the Boxer sequences; however, the exon 5 sequence contained a transition, c.597G>A, which predicts a p.M199I missense mutation (Fig. 3A). Using a pyrosequencing assay that distinguishes the A and G alleles (Fig. 3B), we found that all three of the NCL-affected American Bulldog samples available to us at the initiation of this project were homozygous for the A allele; whereas, the A allele was completely absent in 131 randomly selected dogs representing 108 other breeds. We subsequently obtained and genotyped DNA from 120 additional American Bulldogs including five that exhibited clinical signs of NCL. The frequency of the A allele was 0.28 among the 123 American Bulldogs genotyped in this study. In this group of American Bulldogs, the clinical histories of 66 G/G homozygotes and 45 G/A heterozygotes were unremarkable. Eight of the 12 A/A homozygotes exhibited neurological signs of NCL. The other four A/A homozygotes were less than four months old and had not yet shown signs of the disease.

The amino acid sequence for canine cathepsin D was translated from the canine *CTSD* exon sequences and used to query the non-redundant coding sequence translations in GenBank in a BlastP search for cathepsin D orthologs. As shown in Table 2, the methionine at amino acid position 199 of the canine protein was conserved in the cathepsin

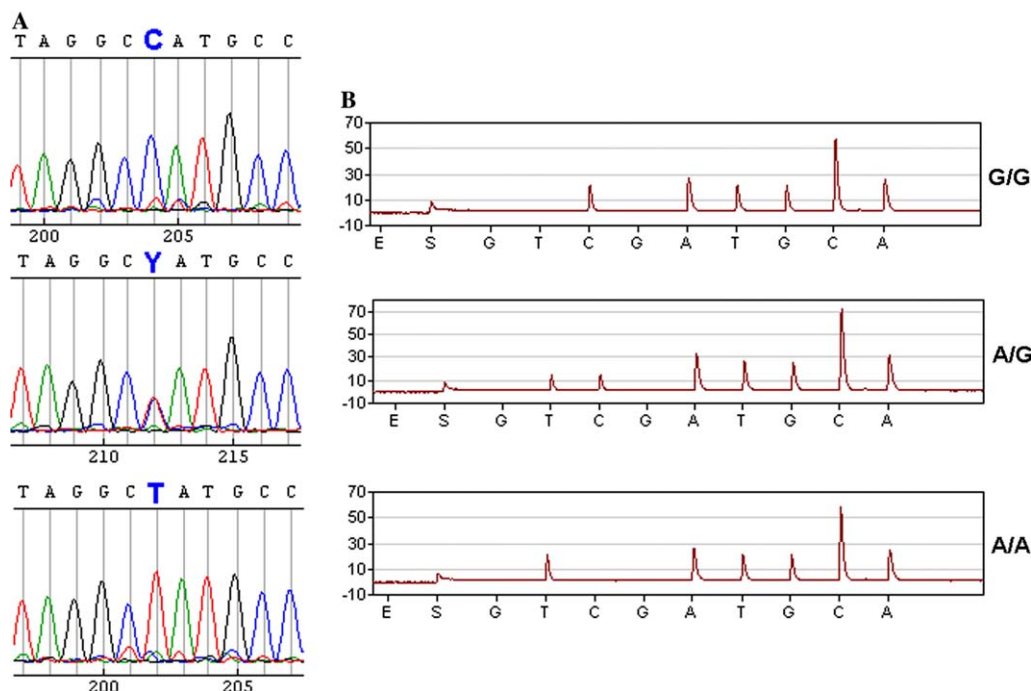


Fig. 3. (A) Aligned sequence chromatograms showing antisense sequence for a segment of *CTSD* exon 5 from an American Bulldog that is homozygous for the wild type or G allele (top), from an A/G heterozygous American Bulldog (middle), and from a NCL-affected American Bulldog that is homozygous for the mutant or A allele (bottom). (B) Pyrosequencing data produced in the antisense direction: from a G/G homozygote (top), an A/G heterozygote (center), and an A/A homozygote (bottom). See Ronaghi [23] for interpretation of pyrosequencing data.

Table 2

Amino acid sequences near methionine-199 of canine cathepsin D and its equivalent in cathepsin Ds from a variety of species

| Amino acid sequence <sup>a</sup>   | Species                                      | Common name              |
|------------------------------------|--|--------------------------|
| IAAKFDGILGMAYPRISVNNV              | 6 species of mammals <sup>b</sup>            |                          |
| ..... G. . F. . . . . <sup>c</sup> | <i>Rattus norvegicus</i>                     | Norwegian rat            |
| V. . . . . G. . H. . . . .         | <i>Mus musculus</i>                          | House mouse              |
| ..... F. . . . . DK.               | <i>Gallus gallus</i>                         | Chicken                  |
| ..... DG.                          | 4 fishes and a salamander <sup>d</sup>       |                          |
| ..... G. . . . . DG.               | <i>Tetraodon nigroviridis</i>                | Green spotted pufferfish |
| ..... A. DG.                       | <i>Silurus asotus</i>                        | Amur catfish             |
| ..... FHIIA. .                     | <i>Sparus aurata</i>                         | Gilt-head seabream       |
| V. . . . . G. . . . . DG.          | <i>Xenopus laevis</i>                        | African clawed frog      |
| V. . . . . G. . K. . . . . DG.     | <i>Xenopus tropicalis</i>                    | Western clawed frog      |
| ..... L. . . . . DK.               | <i>Podarcis sicula</i>                       | Italian wall lizard      |
| ..... T. . . . . K.                | <i>Todarodes pacificus</i>                   | Japanese common squid    |
| ..... T. A. DH.                    | <i>Bombyx mori</i>                           | Domestic silkworm        |
| ..... NS. . . . . DK.              | <i>Drosophila melanogaster</i>               | Fruit fly species        |
| ..... SS. . . . . DG.              | <i>Drosophila pseudoobscura</i>              | Fruit fly species        |
| V. . . . . L. . . . . DG.          | <i>Anopheles gambiae</i>                     | African malaria mosquito |
| V. . . . . LG. SS. . . . . DG.     | <i>Aedes aegypti</i>                         | Yellow fever mosquito    |
| ..... F. E. . . . . LG.            | <i>Ancylostoma ceylanicum</i>                | Hookworm species         |
| ..... F. E. A. LG.                 | <i>Necator americanus</i>                    | New world hookworm       |
| VM. . . . . SLA. GG.               | <i>Schistosoma japonicum</i>                 | Blood fluke species      |
| VM. . . . . S. . . . . DG.         | <i>Schistosoma mansoni</i>                   | Blood fluke species      |
| ..... KT. . . . . DG.              | <i>Clonorchis sinensis</i>                   | Oriental liver fluke     |
| V. . . . . E. A. LG.               | 2 <i>Caenorhabditis</i> species <sup>e</sup> |                          |

<sup>a</sup> Cathepsin D sequences corresponding to amino acids 189–209 in the dog protein.

<sup>b</sup> *Canis familiaris* (Dog), *Homo sapiens* (Human), *Pongo pygmaeus* (Orangutan), *Bos taurus* (Cow), *Ovis aries* (Sheep), and *Sus scrofa* (Pig).

<sup>c</sup> Dot indicates same amino acid as dog and 5 other mammalian species.

<sup>d</sup> *Oncorhynchus mykiss* (Rainbow trout), *Clupea harengus* (Pacific herring), *Takifugu rubripes* (Japanese pufferfish), *Chionodraco hamatus* (Antarctic icefish), and *Hynobius leechii* (Korean salamander).

<sup>e</sup> *Caenorhabditis elegans* and *Caenorhabditis briggsae*.

Table 3  
Enzyme-specific activities in control and NCL-affected dog brains

| Enzyme                  | Specific activity <sup>a</sup> |                         | NCL/control ratio |
|-------------------------|--------------------------------|-------------------------|-------------------|
|                         | Control brains <sup>b</sup>    | NCL brains <sup>c</sup> |                   |
| $\alpha$ -Glucosidase   | 6.5 ± 0.7                      | 11.9 ± 3.2              | 1.84 <sup>d</sup> |
| $\alpha$ -Mannosidase   | 37.5 ± 4.3                     | 48.7 ± 9.1              | 1.27              |
| $\beta$ -Mannosidase    | 4.7 ± 0.6                      | 5.2 ± 0.4               | 1.11              |
| $\beta$ -Galactosidase  | 122 ± 27                       | 157 ± 38                | 1.30              |
| $\alpha$ -Fucosidase    | 11.4 ± 2.2                     | 20.2 ± 5.4              | 1.77 <sup>d</sup> |
| $\beta$ -Glucuronidase  | 45.4 ± 8.6                     | 74.0 ± 14.2             | 1.67 <sup>d</sup> |
| Hexosaminidase-B        | 271 ± 20                       | 453 ± 18                | 1.73 <sup>d</sup> |
| Hexosaminidase-A        | 58.6 ± 7.3                     | 101 ± 18                | 1.91 <sup>d</sup> |
| Acid lipase             | 82.5 ± 20                      | 158 ± 65                | 1.63              |
| Cathepsin B             | 14.7 ± 2.2                     | 30.5 ± 11.9             | 2.07              |
| Cathepsin C             | 139 ± 13                       | 119 ± 13                | 0.86              |
| Cathepsin D             | 2600 ± 263                     | 927 ± 325               | 0.36 <sup>d</sup> |
| Cathepsin H             | 195 ± 23                       | 195 ± 15                | 1.00              |
| Dipeptidyl peptidase II | 19.7 ± 2.9                     | 26.3 ± 4.7              | 1.33              |
| Legumain                | 11.0 ± 1.9                     | 17.6 ± 6.4              | 1.60              |
| Tripeptidyl peptidase I | 57.4 ± 7.6                     | 236 ± 25                | 4.11 <sup>d</sup> |

<sup>a</sup> Enzyme-specific activities in canine brains.

<sup>b</sup> Mean-specific activities in 4 control brains ± SD.

<sup>c</sup> Mean-specific activities in 3 NCL-affected brains ± SD.

<sup>d</sup> Affected activity significantly lower or higher than the control activity ( $P < 0.05$ ).

D sequences of 18 of 19 vertebrates and in all eight mammalian species identified by the query. The one exception among the vertebrates was the Italian wall lizard, *Podarcis sicula*, in which the methionine was replaced by an isoleucine. The BlastP search also identified 13 cathepsin D orthologs from a diverse group of invertebrates, 11 of which had a methionine residue at the corresponding position in the protein. The two exceptions were sequences from mosquitoes, which like the Italian wall lizard, had isoleucine substituted for the methionine.

The mean cathepsin D-specific activity from three brains from NCL-affected American Bulldogs was reduced to 36% of the mean activity from four brains from control dogs

( $P = 0.001$ ); whereas, the affected dog activities of 15 other lysosomal enzymes were either unchanged ( $N = 9$ ) or exhibited a significant increase ( $N = 6$ ; Table 3).

Fig. 4 shows the relationships and genotypes of a 99 member American Bulldog family segregating NCL. We assume that the number of biallelic mutations within the American Bulldog genome is  $N = 3 \times 10^6$ . This estimate is consistent with the 2.1 million single nucleotide polymorphisms detected in the first assembly of the whole genome sequence for dog (<http://www.broad.mit.edu/mammals/dog/>) and leads to an estimate of  $P_0 = 0.99999993156$  if all mutations occur with a minor allele frequency of  $q = 0.27$  and  $P_0 = 0.99999997657$  if mutations are assumed to have minor allele frequencies uniformly distributed between (0,0.5). Consequently, the probability that there is at least one other mutation within the dog genome that is consistent with the pedigree and phenotypes ( $1 - P_0$ ) is less than  $10^{-7}$ .

## Discussion

Previously described mutations in *CTSD* cause early onset NCL in two animal models [8,9]. *CTSD* knockout mice have been studied since 1995 [17]. Nullizygotes appear normal at birth and grow normally for about two weeks, but develop lymphopenia, thromboembolism, and intestinal necrosis leading to death at 25–27 days of age [17]. Koike et al. [9] noted that in the terminal stages of their disease, these mice had seizures and loss of vision and found that neurons in the brains of these mice accumulated cytoplasmic autofluorescent storage granules consistent with NCL.

Tyynela et al. [8] studied sheep with a naturally occurring congenital NCL and identified a missense mutation in *CTSD*, that converted an active site aspartic acid codon to an asparagine codon. The mutant *CTSD* produced an inactive but stable cathepsin D protein. Newborn homozygous affected lambs were weak and unable to stand, but some could suckle and survive for a few weeks with bottle

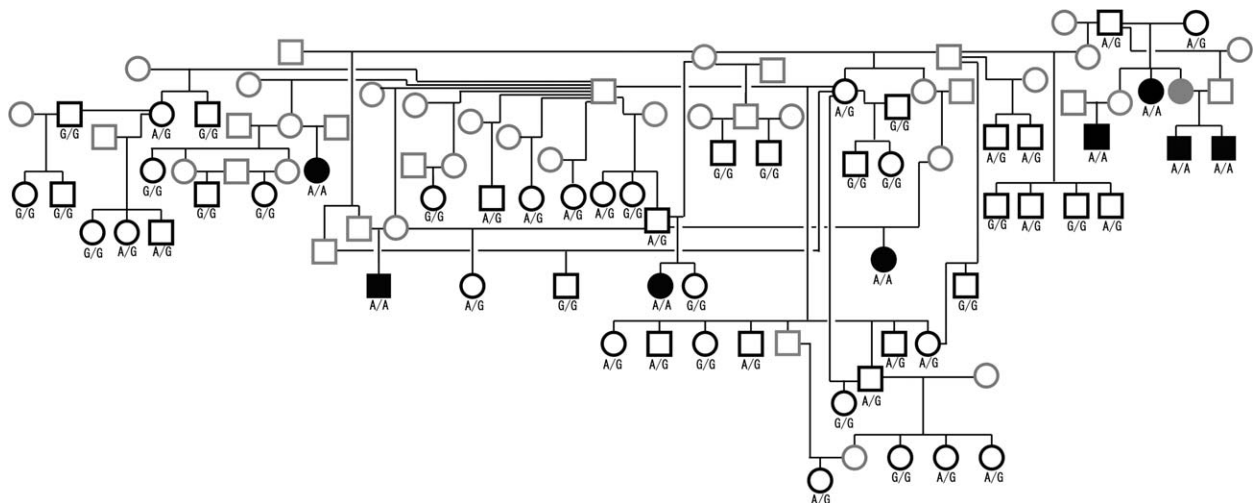


Fig. 4. Pedigree of an American Bulldog family segregating NCL: open squares and circles represent clinically normal individuals; solid squares and circles represent individuals that exhibited clinical signs of NCL. Genotypes at c.597 of *CTSD* are shown below each black symbol; DNA for genotyping was not available from the American Bulldogs represented in gray.

feeding [18]. At birth their brains were grossly underdeveloped and about half normal size [8]. Neurons from these brains contained cytoplasmic autofluorescent storage granules [8,18]. The cells of visceral organs also accumulated storage granules; however, affected sheep did not develop the lymphopenia, thromboembolia, and intestinal necrosis observed in the *CTSD* knockout mice. Brain tissues from homozygous affected sheep lacked detectable cathepsin D activity; whereas, the activities of 12 other lysosomal enzymes were normal or increased in these tissues [8].

Most of the clinical, histological, and ultrastructural characteristics of American Bulldog NCL were described in an earlier report [14]. As in that previous study, we found that autofluorescent lysosomal storage material accumulates in neurons throughout the brains of affected American Bulldogs and in their retinal ganglion cells. In addition, we detected autofluorescent inclusions near the outer limiting membrane of the retina due to the accumulation of storage material in the photoreceptor cells, especially the cones. To our knowledge, there are no previous reports of cytoplasmic autofluorescent inclusions within photoreceptor cells in conjunction with any of the human or animal NCLs. If this photoreceptor pathology is, in fact, unique to NCL caused by *CTSD* mutations, it would imply that cathepsin D is essential for normal protein turn over in photoreceptors; whereas, there is no comparable requirement for those gene products that are abnormal or missing in the other NCLs. It is not yet known whether the accumulation of storage material in the photoreceptors is associated with impaired photoreceptor cell function.

The ultrastructural appearance of the cytoplasmic inclusions in most previously described human and animal NCLs have been classified as curvilinear, fingerprint, or reticular profiles or as granular osmophilic deposits [19]. Unlike these classical ultrastructures, the storage bodies in the American Bulldog disease observed here and in an earlier study [14] consisted of round uniformly staining inclusions embedded within granular matrixes. They resemble the inclusion body ultrastructures from the *CTSD* knockout mice [8] and certain atypical human NCLs [20].

An adult-onset neurodegenerative disease with some features of NCL was recently reported in two breeds that are closely related to the American Bulldog: the American Staffordshire Terrier and the Pit Bull Terrier [21]. In the latter breeds, neurodegeneration is accompanied by the accumulation of cytoplasmic autofluorescent storage material; however, the storage material accumulation and neurodegeneration were found only in cerebellar Purkinje cells and in certain thalamic nuclei. This restricted distribution of lesions differs greatly from the wide-spread distribution, which is observed in brains from affected American Bulldogs [14] and which is characteristic of all previously described NCLs. Furthermore, curvilinear and fingerprint profiles are prominent ultrastructural features of the storage material in the American Staffordshire/Pit Bull Terrier disease [21]; whereas, the American Bulldog storage mate-

rial has the atypical granular ultrastructure described above. Thus, the American Bulldog NCL is distinct from the neurodegenerative disease described in American Staffordshire and Pit Bull Terriers.

We have identified a G to A transition within the canine *CTSD* gene. Although, the A allele appears to be rare or absent in other breeds, it was found in 28% of the chromosomes from the 123 American Bulldogs genotyped in this study. Eight of these American Bulldogs exhibited clinical signs of NCL and all eight were A/A homozygotes. The four other A/A homozygous American Bulldogs were all less than four months of age and presumed to be preclinical. Since, DNA samples were not available from many of the siblings and ancestors of the affected dogs, we were unable to perform a linkage analysis to demonstrate a two-point association between the disease locus and the c.597G > A mutation. Instead, we performed a transmission analysis in which we estimated the probability that a randomly sampled biallelic mutation would be concordant with the pedigree and known phenotypes of dogs aged two years old or older. From this probability, we estimated the probability that there would be at least one mutation unlinked to *CTSD* in the genome concordant with the pedigree and phenotypes and demonstrated that this event was extremely unlikely. We therefore conclude that, there is strong evidence supporting the identity of c.597G > A, or a tightly linked mutation, as the cause of NCL in American Bulldogs.

The c.597G > A transition is a missense mutation in exon five of canine *CTSD* that converts methionine-199 to an isoleucine. Methionine-199 in the canine cathepsin D amino acid sequence is preceded by an eight amino acid sequence, AKFDGILG, that is conserved in cathepsin D sequences from all of the other 31 diverse species identified by a BlastP search of the non-redundant translated GenBank sequences (Table 2). This eight amino acid motif is followed by methionine in 29 of the 32 cathepsin D sequences. The three exceptions are from a lizard and two species of mosquito; and, in all three exceptions the amino acid that replaces the methionine is isoleucine. Canine cathepsin D is an aspartate protease and aspartic acids at positions 97 and 293 of the canine protein contribute to the active site [22]. The functional role of methionine-199 and nearby amino acids is unknown; however the conservation of the adjacent amino acid sequence across every species for which sequence was detected implies that the region is under strong purifying selection and thus, has a functional role.

The substitution of isoleucine for methionine at position 199 is a conservative change; however, the association of this mutation with NCL suggests that this substitution may affect the biosynthesis, subcellular targeting, or stability of cathepsin D. Brain tissues from homozygous A/A American Bulldogs had a significant decrease in cathepsin D activity to 36% of that in control dog brain tissue. It is unlikely that this activity reflects contributions from enzymes other than cathepsin D, because the identical assay procedure failed to

detect any activity in brains from sheep homozygous for a cathepsin D missense mutation involving an active site aspartate [8]. Fifteen other lysosomal enzyme activities in affected brains were either similar to normal ( $n=9$ ) or significantly increased ( $N=6$ ; see Table 3). This apparent compensatory upregulation of other lysosomal enzymes in response to a cathepsin D deficiency was also noted in brain tissue from the sheep with congenital NCL [8] and is typical of lysosomal storage disorders.

In contrast to the NCLs of sheep and mice, which lack detectable cathepsin D activity [8,9], brains from homozygous affected American Bulldogs retained 36% of the cathepsin D activity found in control dog brains. This appears to be comparable to the reduction to 40% of normal cathepsin D activity, which was reported for fibroblasts cultured from clinically normal sheep that are heterozygous for the ovine *CTSD* mutation [8,18]. It is not clear that a reduction in an enzyme activity in brain sections from one species can be validly compared to a reduction in that enzyme activity in fibroblast cultures from another species. Furthermore, it is uncertain by what criteria or at what age the heterozygous sheep were evaluated for symptoms of disease. Nonetheless, it is surprising that the 36% residual cathepsin D activity did not protect the homozygous American Bulldogs from developing lethal NCL. It is possible that abnormal subcellular targeting or other aberrations prevent affected dogs from fully benefiting from the residual cathepsin D activity detected in their brains. On the other hand, the clinical manifestations of American Bulldog NCL appear to be substantially less severe than those endured by the sheep or mice that completely lack cathepsin D activity. Thus, the residual cathepsin D activity in the dogs is likely to be of some benefit in moderating the symptoms.

Human cathepsin D deficiency diseases have not yet been described. Characteristics of the cathepsin D deficiencies of sheep and mice suggest that the human disease might present as a congenital NCL. The American Bulldog disease indicates that human cathepsin D deficiency might also present as a later onset disease. Whether or not human cathepsin D deficiency diseases exist, the American Bulldog NCL may be a useful model for those developing gene or enzyme replacement therapies for lysosomal storage diseases affecting the brain.

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