DISCOVERING DISEASE CAUSING VARIANTS IN DOGS THROUGH WHOLE GENOME SEQUENCING

A Dissertation presented to the Faculty of the Graduate School
University of Missouri

In Partial Fulfillment
Of the Requirements for the Degree

Doctor of Philosophy in Veterinary Pathobiology

by

Ana Leticia Kolicheski

Dr. Gary S. Johnson, Dissertation Supervisor

July 2017

The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled DISCOVERING DISEASE CAUSING VARIANTS IN DOGS THROUGH WHOLE GENOME SEQUENCING, presented by Ana Leticia Kolicheski a candidate for the degree of Doctor of Philosophy in Pathobiology and hereby certify that in their opinion it is worthy of acceptance.

Dr. Gary S. Johnson

Dr. Bimal K. Ray

Dr. Leslie A. Lyons

Dr. Christine G. Elsik

Dr. Robert D. Schnabel

Acknowledgements

First and foremost I would like to thank Dr. Gary S. Johnson for giving me the opportunity to work in his lab and guiding me through this PhD program. Dr. Johnson is an amazing mentor, not only for the free meals and frogs, but he made a permanent mark in my life with his kind nature, quirky and dry sense of humor, patience, hard work and perseverance. I would like to express my gratitude to my dissertation committee, Dr. Bimal K. Ray, Dr. Leslie Lyons, Dr. Christine G. Elsik, and Dr. Robert D. Schnabel for all the helpful comments and guidance throughout my program. Each of you has had a very positive impact on me. I am thankful to have learned so much from you and your different areas of expertise; those are useful skills that I will carry throughout my professional life.

I would also like to thank all the lab crew: Dr. Tendai Mutangadura, Dr. Juyuan Guo, Liz Hansen, Peggy Ann Eichen, Natalie Villani, and the so many other members that have come and gone throughout the years. I also acknowledge Dr. Dennis O'Brien, Dr. Martin Katz, Dr. Joan Coates, and the many other research collaborators. I am also extremely thankful for all the support and encouragement I received from all of my family. My family was always there for me and helped to motivate me through the hard times, even from far away. A special thank you to my lovely boyfriend Thomas Stauffer, and to my adoptive American family: Donna and Don Stauffer, and Brian and Alexandria Stauffer, who were always supportive and believed in me.

Last but not least, I would like to thank my friends in Columbia, Guilherme Hosotani, Mariana Masiero, Estela Kobashigawa, Francine Russo, Brock Duck, Tim Poindexter, Sidharth Sen, Barbara Gandolfi and Erica Creighton. And my friends who are far away and still being supportive, Nathaly Gasparin, Mieke Stevens, Stefan Norenus, Brian Davis and Fabiana Farias.

Table of Contents

Acknowledgements	ii
Abstract	v
Chapter 1	1
Introduction	1
Chapter 2.....	16
A homozygous <i>PIGN</i> missense mutation in Soft Coated Wheaten Terriers with a canine paroxysmal non-kinesigenic dyskinesia	16
Abstract.....	16
Introduction	17
Methods	19
Results	22
Discussion.....	27
Chapter 3.....	42
Australian Cattle Dogs with Neuronal Ceroid Lipofuscinosis are Homozygous for a <i>CLN5</i> Nonsense mutation Previously Identified in Border Collies	42
Abstract.....	42
Introduction	43
Materials and Methods.....	45
Results	54
Discussion.....	61
Footnotes	65
Chapter 4.....	66
A homozygous <i>PPT1</i> splice donor mutation in a Cane Corso Dog with neuronal ceroid lipofuscinosis	66
Abstract.....	66
Case Report.....	67
Supplemental material	85
Footnotes	88
Chapter 5.....	89

GM2 gangliosidosis in Shiba Inu dogs with an inframe deletion in <i>HEXB</i>	89
Abstract.....	89
Introduction	90
Methods	91
Clinical Findings.....	91
Molecular-genetic diagnosis.....	94
Biochemical and electron microscopic confirmation.....	95
Autofluorescent storage bodies in NCL and other diseases.....	101
Comparison with other gangliosidoses in dogs	103
Conclusions	103
Supplemental Material	105
Chapter 6.....	110
Discussion and Conclusion	110
References	127
VITA	151

Abstract

This dissertation focuses on the use of whole genome sequencing (WGS) for the identification of disease causing variants in canine genomes. A brief review on the historical milestones of genetics, the creation and popularization of the fast throughput DNA sequencing technologies and their advantages and potential problems and biases, the importance of the study of canine genetics and the current state of the canine genome assembly is presented. Our lab sequenced ~100 dogs in the attempt to discover disease-causing variants. So far 20 such variants have been identified. This dissertation contains detailed accounts of the discovery variants likely to be responsible for four canine diseases. Those diseases are: Paroxysmal dyskinesia in Soft Coated Wheaten Terriers that is associated to the missense mutation *PIGN:c.398C>T*; two different forms of neuronal ceroid lipofuscinosis, one in Australian Cattle dogs caused by *CLN5:c.619C>T*, and one in the Cane Corso caused by the splice site mutation *PPT1c.124+1G>A*; and a Shiba Inu GM2 gangliosidosis caused by *HEXB.c:948_950delCCT*. Furthermore, examples of not so successful attempts, possible reasons for failures and suggestions to successfully conclude other ongoing investigations.

Chapter 1

Introduction

Next-generation sequencing (NGS) is one of the current technologies used for genetic variation screening and novel variant discovery for personalized medicine. Due to decreased costs and fast turnaround, NGS is also becoming viable option for personalized veterinary medicine. This dissertation focuses on the use of whole genome sequencing (WGS) for the identification of disease-causing variants in canine genomes. This introductory section reviews important milestones in the history of genetics and sequencing technologies. In addition it describes how the newer technologies have contributed to the establishment of personalized medicine and how WGS is contributing to the study of canine genetics and inherited canine diseases.

Genetics as a science and formal discipline is relatively young historically. Mankind, however, has been wondering about the origins of life, and how some traits are transmitted through generations since the Greek ancient times. For example, Herodotus produced one of the first known descriptions of a genetic condition in humans in one of his book passages: “The goddess [Aphrodite] afflicted the Scythians... and all their descendants forever with hermaphroditism...”¹. Then attributed to Gods, curses, and the supernatural, several familial diseases, such as epilepsy, dwarfism, hemophilia, color blindness, polydactyly and albinism, were anecdotally described by ancient physicians.¹ The

scientific community formally started to document their philosophies and theories regarding genetics in the early 1800s, more specifically when Jean-Baptiste Lamarck wrote the first coherent and occasionally still discussed theory of evolution.^{2,3} In 1858 Charles R. Darwin published the widely accepted concepts of natural selection and evolution.⁴ Simultaneously, Gregor Mendel described “Mendelian inheritance” based on his observations while breeding pea plants.^{5,6} In 1871 Friedrich Miescher was the first scientist to isolate DNA from the nuclei of white blood cells, which he then called “nuclein.”^{7,8} The significance of “nuclein” was unrecognized until much later, when in 1944 the link between the DNA molecule and heredity was established by Avery, MacLeod and McCarty.^{6,9} Scientists world-wide became excited with the discovery and the “DNA era” began. The structure of DNA was described by James Watson and Francis Crick in the 1950s.^{6,10} Their discovery was based on the X-ray crystallographic investigations by Rosalind Franklin and Maurice Wilkins,^{10,11} together with the previous knowledge generated through biochemical studies of nucleotides by Phoebus Levene^{10,12} and Erwin Chargaff.^{10,13,14} Twelve years later, scientists could sequence nucleotides through fragmenting RNA into short oligonucleotides with various RNase enzymes and then identify the residues using two-dimensional chromatography and spectrophotometry. With this extremely laborious methodology, only a few base pairs per year could be sequenced, not nearly enough to sequence an entire gene.¹⁵ In 1977, Sanger and Maxam and Gilbert developed novel sequencing methods.^{16,17} Sanger’s method prevailed over the Maxam and Gilbert’s method because it is simpler, more reliable and used fewer

toxic chemicals.¹⁸ Polymerase chain reaction (PCR) was invented by Kary Mullis in 1983, when he was working for Cetus Corp as a chemist. Mullis won the Nobel prize for inventing PCR in 1993.¹⁹ The invention of PCR technology allowed the automation of Sanger sequencing and revolutionized the genetics field, and the establishment of GenBank (<http://www.ncbi.nlm.nih.gov/genbank>) resulted in the generation of thousands of sequences through the 1980s, 1990s and early 2000s.^{18,20-22} Automated Sanger sequencing is still widely used for sequencing short segments of DNA and confirming genotypes from other technologies, among other applications.^{20,21,23} Many different fields currently benefit from the different methodologies of nucleotide sequencing and genotyping including: forensics sciences, evolution research, medical research, cloning, genetically modified organisms, gene therapy, animal breeding, crop improvement, and environment conservation genetics, among others.

The first complete human whole genome sequence was done by the International Human Genome Sequencing Consortium, funded by the National Human Genome Research Institute (NHGRI). The human genome project (HGP) took 13 years for completion (1990-2003) and had a cost of over 2.7 billion dollars.²⁴ A second human genome sequence was released nearly simultaneously by the Celera Corporation, led by Craig Venter and colleagues.²⁵ Those sequences were generated with slightly different “shotgun sequencing” methods. Shotgun sequencing consists in random sequencing of sheared genome fragments, for a later contig construction. The HGP opted for a “hierarchical shotgun sequencing” approach that involved the generation and organization of a set of large-insert

bacterial artificial chromosomes (BACs) clones (100-200kb) that cover the genome separately and in an ordered manner, followed by the shotgun sequencing and assembly of the BAC-clones hierarchically, as an attempt to diminish the risk of misassembly.²⁴ Celera's group used a mixed strategy, involving some coverage with whole-genome shotgun data together with the publicly available hierarchical shotgun data generated by the International Human Genome Sequencing Consortium.²⁵ HGP's assembly process involved filtering, to uniformly eliminate contamination from nonhuman sequences and other artifacts, placing the sequences on the physical map, and merging the sequences generating sequence contigs.²⁴ The merged BAC sequences had fairly large contigs (half with over 80 kb) and represented ~90% of the euchromatic portion of the human genome, with a total sequence coverage of 7.5-fold. Celera's assembly strategy was a "joint assembly" of Celera's WGS data (5.1-fold coverage) and HPG's data. The Celera's joint assembly output did not contain any dramatic differences other than adding a few percent additional sequence and ordering information, however, their approach demonstrated that it is possible to use whole-genome shotgun sequencing for assembling large genomes, which is much faster, easier and cost-effective than the hierarchical shotgun sequencing methodology.²⁶

High throughput massively parallel sequencing technologies started to appear around 2005. These technologies substantially reduced sequencing times and costs. Since then, the world has been experiencing a booming "genomic era."¹⁸ The impressive advances of the "genomics era" can be attributed to the concomitant rapid technological advances in the fields of computer science,

computer engineering, biochemistry and bioengineering. Nowadays, a human genome can be sequenced with ~30X average coverage in just a few days, for a total cost of ~\$2000 with the newest DNA sequencing technologies, and in the future a genome may possibly be sequenced for about \$1,000 or less in about 15 minutes.²⁰ The current challenges involve data storage, interpretation and integration, which are further discussed in this chapter.

Due to the increased sequencing reaction speed, cost reduction and technical ease, the amount of genomic data is rapidly increasing and has become popular in interspecies research. Currently, genomes from ~277 different vertebrate species have already been sequenced and there is an ongoing “Genome 10k project” that aims to assemble a collection of DNA sequences representing the genomes of ~10,000 vertebrate species.^{27,28} There is an estimate is that by the year 2025, researchers will be producing between 2 and 40 Exabyte of genomic data per year.²⁹ The more good quality data available, the more accurate the cumulative databases will become, which will facilitate data interpretation in the future.

The dog is among the many species with a sequenced reference genome, and is a very important biomedical animal model.³⁰ Because dogs mostly share the same environment as humans and have a relatively similar physiology, the study of the dog genome can provide valuable information about our own evolutionary and immigration history and health.³¹ In addition, these studies may lead to the discovery of novel genetic pathways that contribute to disease and other phenotypes, such as body size and skull shape.³² Dog genome studies can also

lead to the development of DNA tests that are powerful diagnostic tools, which can help breeders eliminate undesired traits.^{30,33}

Many details about the origin of the domestic dog origin are still under debate, such as place of origin (Europe or Asia) and the number of domestication events.³⁴ The consensus among experts is that dog domestication occurred sometime between ~15,000-100,000 years ago and involved several levels of admixture between different domestic dogs and wolves.^{31,35-38} Breed formation took place more recently and currently ~350 registered dog breeds exist. Not surprisingly, genetic evolutionary and population studies revealed that the canine species has a very unique population structure and an extreme genome plasticity,³⁹ which is explained by the two major genetic bottlenecks, domestication and breed formation, that are responsible for shaping the current canine haplotype structure and linkage disequilibrium (LD). Intra-breed LD in dogs can extend for several Mbs, meaning individuals of the same breed are relatively genetically homogenous, whereas the interbreed LD extends for only a few kbs. The canine haplotype and LD structure bring advantages for genome-wide association studies (GWAS), as regions associated to certain traits (mostly Mendelian) can be detected using fewer markers than required for other species, such as humans, which have shorter LD³⁹

The first canine whole genome assembly was generated in 2003 through whole-genome shotgun sequencing of a male standard poodle, with an average of 1.5X coverage (6.22 million reads), which covered 77% of the coding genome of a sequence of four overlapping BACs that provided a 512 kb reference of the dog genome, with 265 gaps of 452 bases mean length.⁴⁰ In 2005, a female boxer with

a high degree of homozygosity was selected and sequenced for an improved version of the canine genome reference sequence by the Broad Institute.⁴¹ A whole-genome shotgun sequencing approach was used to produce a reference sequence that spanned a total of 31.5 million reads providing ~7.5X coverage. The first assembly (CanFam 1.0) was generated using an updated version of the ARACHNE program.⁴² An updated assembly (CanFam 2.0) had minor improvements, such as longer contigs and fewer genic gaps. The sequence contigs of the boxer sequence were ~50-fold larger than the standard poodle sequence, and 97% of the assembled sequence was anchored to the canine chromosomes. However even with the improvements and high quality, ~1% of the euchromatic genome resided in gaps and ~20% of genes show some evidence of sequencing errors, such as frameshifts, very short introns, and missing first exons and promoters. The most current build (CanFam3.1) improved the quality and completeness of the canine genome sequencing by closing the small gaps with 12,101 primer walks, and closing the larger gaps and correcting mis-assembled regions through the re-sequencing of targeted BACs. In total, 85 Mb of novel sequence was integrated into CanFam2.0 to create CanFam3.1. The new assembly (CanFam3.1) covers 99.8% of the euchromatic portion of the genome, and it also provides a much improved annotation due to RNA-sequencing data sets from 10 different canine tissues.⁴³

The early canine gene mapping studies were done by linkage analysis with sparse numbers of microsatellite markers.⁴⁴ With the availability of the high quality draft of the canine reference sequence, microsatellite technology was rapidly replaced by

high density SNP arrays.³⁰ Linkage mapping and GWAS research have been effective in identifying the chromosomal locations of genes responsible for many Mendelian traits, including diseases. One of the first successes for identification of a disease-linked marker occurred in 1997 with the detection of a microsatellite linked to the canine copper toxicosis in Bedlington Terriers.⁴⁵ Other successful microsatellite studies identified the first canine epilepsy gene (a canid-specific unstable dodecamer repeat in *EPM2B*)⁴⁶ and the cause of dwarfism in German Shepherds (deletion of one of six 7 bp repeats in intron 5 of *LHX3*).⁴⁷ GWAS studies using high density SNPchips and the improvement of the reference sequence increased the resolution and sensitivity of the mapping and also allowed researchers to use unrelated animals in their experimental design. Well known successes with this technology include identification of the cause of the hair ridge in Rhodesian ridgebacks,⁴⁸ the finding that hair coat variation that is determined mostly by 3 genes (*RSPO2*, *FGF5*, and *KRT71*)⁴⁹ and the discovery of a mutation in *SOD1* that is a risk factor to degenerative myelopathy in many breeds,⁵⁰ among many other important studies.

While the use of high density chips for GWAS mapping is still relevant for disease locus discovery, whole genome sequencing via NGS is becoming the preferred technology. In some instances a whole genome sequence from a single affected individual has led to the identification of causal variants because previously generated sequences from individuals from different breeds and different disease phenotypes have been used as controls.⁵¹⁻⁵⁵

Next-generation sequencing refers to high throughput sequencing technologies and includes multiple sequencing platforms, e.g. Illumina, Inc,⁵⁶ Life Technologies Corp,⁵⁷ Pacific Biosciences, Inc.,⁵⁸ among others. The Illumina platform is the most commonly used for canine genetics research due to cost benefits. Typically, the average read length of the Illumina platform is ~100 bp per read pair with an average ~30X coverage along the genome, which is enough to identify a good portion, but unfortunately not all, genome variation. The shorter read lengths may not align to a reference genome uniquely in repetitive regions, so interpreting copy number variation and large deletions and insertions can be challenging. Longer read lengths are desirable for detecting such large genome variations, however the current long read technologies are not yet cost effective for mass genotyping, and their use is so far limited to de novo assemblies.⁵⁹

While sequence data generation has become routine, data analysis and interpretation remain challenging. The creation of databases and pipelines are essential for quality research. In general, the steps for NGS data analysis include data tracking, barcoding, storage, quality control, sequence alignment to the reference genome, variant calling and interpretation. Data tracking pertains to good practices in maintaining databases and linking genotype data to a certain individual and phenotype information. Barcoding assures that the correct output is attributed to the correct sample when multiplexing different samples and libraries. Barcodes are unique DNA sequences of approximately 8-12 bp that are ligated to fragments within the sequencing library, and can be identified and sorted using bioinformatics software.⁶⁰

Data storage is an important concern because genomes are large, therefore the sequencing files are also large. For example, a canine WGS with an average of 30X coverage uncompressed FASTQ file has typically ~100-180 GB, which is why compressing FASTQ files for storage is helpful. Sequence Alignment Map (SAM) format is the aligned FASTQ file, and contains more information than the raw FASTQ, and its usually compressed into a Binary Alignment Map (BAM) format. This can reduce the file size to ~30-50 GB.⁶¹ The researcher needs to consider the raw data file sizes and add extra room for intermediate processing files in order to make the right choices when purchasing the hardware to store their data.

The researcher must be cautious to avoid possible biases that can be introduced in multiple steps of the data generation and analysis. Sample bias can be avoided by assuring that the sample contains undegraded DNA of sufficient concentration (10-20µg), is free of contaminants, and that the EDTA concentrations not higher than 1mM.⁶² The method of library preparation can be either PCR-based or PCR-free. The PCR-based methods tend to introduce PCR amplification bias (over representing the more efficiently amplified sequences) with lower coverage in high GC% and repetitive content regions.⁶³ Mispriming events may also occur in PCR-based libraries, which can then introduce false positive variation in certain regions of the genome, especially in samples with low DNA quantity or few sequencing reads.⁶⁴ PCR-free libraries can eliminate most of the coverage biases introduced by PCR-based libraries. However, very high GC-rich areas (GC% ≥ 75) can still be problematic.⁶⁵ There are a few downsides to the use of PCR-free libraries that can be easily overcome. For example, the library preparations require relatively large

amounts of sample DNA, that might not be feasible for some sample types, and the method is more sensitive to variations in DNA quality and purity. Because the PCR-free protocols are harder to automate, high-throughput sequencing facilities do not opt PCR-free as the default protocol.⁶³ The library quality control (QC) consists of verifying if the insert sizes are as expected and if there are contaminating adapter-dimers.

In addition to coverage bias, different NGS technologies can introduce chemistry error biases, especially in regions adjacent to long homopolymers, GC extremes, deletions and insertions.⁶⁵ Because of the possible base calling errors, Phred quality scores are generated for each sequenced base. Phred quality scores or Q are logarithmically related to base-calling error probabilities and should be generally above Q30 (chances that the base is called incorrectly is 1 in 1000). Reads with low overall Q values are usually discarded from the analysis.⁶⁶ There are many different choices for quality control (QC) software and metrics that can be used to guarantee the data is useful during multiple steps of the pipeline (raw data, aligned data and variant calling). The researcher should evaluate which one is the most relevant for their specific type of data.⁶⁷ Further details about some of those different methods are discussed in chapter 6 as personal experiences.

Even though there are still issues, NGS is becoming the gold-standard for clinical cytogenetics and personalized medicine.⁶⁸ For example, whole exome sequencing has been used in human medicine as an alternative method for cost-effective clinical research, e.g. the genetic diagnose for Bartter syndrome,⁶⁹ autoimmune lymphoproliferative syndrome (ALPS)⁷⁰ and childhood inflammatory bowel

disease.⁷¹ Recent association studies found that >80% of disease-associated sequence variants fall outside of coding regions^{72,73} and targeted exome sequencing can miss important regulatory variation. Therefore, whole genome sequencing (WGS) is now more cost effective and is becoming routine in the clinical practice and personalized medicine.^{74,75} WGS can be successfully used to alter the treatment plan of a patient with cancer⁷⁶ and identify novel disease-relevant preclinical risk factors for complex diseases such as cancer.⁷⁷ It can also be used as a non-invasive method to detect organ transplant rejection⁷⁸ and detect fetal Down syndrome and other fetal aneuploidies.⁷⁹

The benefits of WGS for the identification of disease causing variants can also be translated to veterinary medicine, and several canine genome researchers are using this technology. Chapters 2 to 5 describe examples of 4 successes achieved with this effective methodology, in the order of their discovery. Chapters 2 through 4 are copies of the reports as published in peer-reviewed journals. The Manuscript in chapter 5 has been accepted after peer review and modification by the copy editor. This draft has received final editorial acceptance. Chapter 6 is a discussion of the overall research efforts during my graduate studies at the University of Missouri and concluding remarks about these research efforts.

Chapter 2 has been published in Neurogenetics (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5243907/pdf/10048_2016_Article_502.pdf).⁸⁰ This chapter describes a paroxysmal dyskinesia in Soft Coated Wheaten Terriers caused by a nonsynonymous variant in *PIGN*. For this publication, my role was to validate the candidate allele *PIGN*:c.398C>T using a

PCR-restriction fragment length polymorphism (PCR-RFLP) assay and then to genotype ~1200 animals using an allelic discrimination assay.⁸¹ I also assisted with the writing of the molecular genetics part of the Materials and Methods and generated the Supplemental Tables and Figure.

Chapter 3 has been published in the Journal of Veterinary Internal Medicine (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5084771/pdf/JVIM-30-1149.pdf>).⁸² This article describes the clinical histories of four Australian Cattle Dogs with neuronal ceroid lipofuscinosis (NCL). All four dogs were homozygous for a nonsense mutation in *CLN5*, which had been previously identified as a cause of NCL in Border Collies. This was the first report that the same variant allele was responsible for NCL in more than one breed. For this study, I processed the WGS data for the affected dog and 43 control dogs through the data analysis pipeline. I also aligned the error corrected reads to the canine reference sequence and generated the variant reports. In addition, I sequenced the four affected Australian Cattle Dogs by direct Sanger sequencing of a variant-containing PCR amplicon and used a TaqMan allelic discrimination assay to genotype over 700 dogs for the variant allele. In addition, and I wrote the first draft of the Materials and Methods and generated Table 2.

Chapter 4 is published in the Journal of Veterinary Internal Medicine (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5259623/pdf/JVIM-31-149.pdf>).⁵³

This chapter contains a case report of a NCL in a single female Cane Corso dog. The diagnosis was based on clinical signs and results from light, fluorescence and electron microscopy. A splice donor mutation in a known NCL gene

(*PPT1c.124+1G>A*) was identified as the probable disease-causing variant by whole genome sequencing. For this report, I identified the causal mutation in the whole genome sequence by aligning the affected dog sequence reads to the canine reference sequence and visually inspecting the sequences of the 13 known NCL-causing genes using a genome browser (IGV). I used an online tool (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html) to estimate the splice donor strength of the nucleotide sequences surrounding the canonical splice site. I also validated the mutation using Sanger sequencing of a variant-containing PCR amplicon. In addition, I designed a nested primer, two-stage allelic discrimination assay to genotype for the causal splice donor variant which was located in a particularly difficult chromosomal region due to the highly repetitive and GC-rich content. I used this two-stage allelic discrimination assay to genotype a cohort of Cane Corso samples. I wrote the first draft of the Materials and Methods section, the Supplemental material and table, and generated the tables in the text and Figure 6.

Chapter 5 is the text of a peer reviewed and accepted article for the Journal of Veterinary Internal Medicine. This case report describes two Shiba Inu Dogs with clinical signs and autofluorescence consistent with NCL. The individual cases were referred to us from independent sources. Based on clinical signs and the finding of autofluorescent storage bodies in affected-dog neurons, the initial diagnosis was NCL. However, WGS failed to identify a candidate variant in a known NCL-associated gene. Instead, a 3 bp inframe deletion in *HEXB* was identified in a whole genome sequence from one of the affected Shiba Inu, suggesting a

probable diagnosis of GM2 gangliosidosis. This diagnosis was confirmed by thin layer chromatography and enzymology. For this report I generated and analyzed the variant reports for the affected dog and other 105 whole genome sequences used as controls, identified the *HEXB.c:948_950delCCT* variation, and confirmed the 3 base pair deletion through PCR-RFLP. In addition, I wrote the first draft of the paper and generated the supplemental table and figure.

Chapter 6 is the discussion and conclusion segment of this dissertation. In this chapter, different methods, pros, cons, future prospects and challenges of WGS analysis and its applications in personalized veterinary medicine is further discussed. My personal experiences with the data analysis and interpretation, and hypotheses on ways to increase the success of causal variant discovery rate are also discussed.

Chapter 2

A homozygous *PIGN* missense mutation in Soft Coated Wheaten Terriers with a canine paroxysmal non-kinesigenic dyskinesia

Abstract

Hereditary paroxysmal dyskinesias (PxD) are a heterogeneous group of movement disorders classified by frequency, duration, and triggers of the episodes. A young-adult onset canine PxD has segregated as an autosomal recessive trait in Soft-Coated Wheaten Terriers. The medical records and videos of episodes from 25 affected dogs were reviewed. The episodes of hyperkinesia and dystonia lasted from several minutes to several hours and could occur as often as >10/day. They were not associated with strenuous exercise or fasting but were sometimes triggered by excitement. The canine PxD phenotype most closely resembled paroxysmal non-kinesigenic dyskinesia (PNKD) of humans. Whole genome sequences were generated with DNA from 2 affected dogs and analyzed in comparison to 100 control canid whole genome sequences. The two whole genome sequences from dogs with PxD had a rare homozygous *PIGN*:c.398C>T transition, which predicted the substitution of an isoleucine for a highly conserved threonine in the encoded enzyme. All 25 PxD-affected dogs were *PIGN*:c.398T allele homozygotes, whereas there were no c.398T homozygotes among 1185 genotyped dogs without known histories of PxD. *PIGN* encodes an enzyme involved in the biosynthesis of glycosylphosphatidylinositol (GPI), which anchors a variety of proteins including CD59 to the cell surface. Flow cytometry of *PIGN*-knockout HEK239 cells expressing recombinant human *PIGN* with the c.398T

variant showed reduced CD59 expression. Mutations in human *PIGN* have been associated with multiple congenital anomalies-hypotonia-seizures syndrome-1 (MCAHS1). Movement disorders can be a part of MCAHS1, but this is the first PxD associated with altered GPI anchor function.

Introduction

The human paroxysmal dyskinesias (PxD) are a heterogeneous group of diseases characterized by episodes of abnormal involuntary movements.⁸³ The episodes may last <1 min or continue for many hours. Episode frequency may vary from <1/year to 100/day. The abnormal movements can be dystonia, chorea, athetosis, or ballism, either singly or in various combinations.⁸⁴⁻⁸⁶ Many PxD episodes are initiated by a recognized trigger, and the type of trigger has served as a means of subclassifying PxD.^{83,84,87} In paroxysmal exertional dyskinesia (PED), the episodes are triggered by sustained exercise. Sudden movements trigger the episodes in paroxysmal kinesigenic dyskinesia (PKD). In paroxysmal non-kinesigenic dyskinesia (PNKD), stress, fatigue, and hunger can trigger episodes in some patients, but the most consistent triggers are alcohol or caffeine consumption. Other factors considered in classification are age of onset, frequency and duration of the episodes, and response to therapy.^{83,84,87-89}

PxD may be secondary to a specific etiology such as encephalitis or stroke⁹⁰ or idiopathic. The latter can be sporadic or familial, usually with an autosomal dominant inheritance.⁸⁷ Mutations in three genes, *SLC2A1*, *PRRT2*, and *PNKD* (also referred to as *MR-1*), are well-recognized causes of PxD.^{87,91-95} *SLC2A1*

encodes glucose transport protein 1 (GLUT1), which facilitates glucose transfer across the blood-brain barrier. Most patients with heterozygous *SLC2A1* mutations exhibit GLUT1-deficiency syndrome characterized by low CSF-to-blood glucose ratios together with epilepsy, developmental delay, microcephaly, ataxia, and PxD.⁹⁶ A minority of patients with heterozygous *SLC2A1* mutations develop isolated PED.^{93,97-99} *PRRT2* encodes proline-rich transmembrane protein 2, which interacts with SNAP25 and thereby influences the release of glutamate or other neurotransmitters.¹⁰⁰ Heterozygous *PRRT2* mutations have been found in patients with several paroxysmal neurologic disorders including PKD.¹⁰¹ Many PNKD patients carry heterozygous mutations in *PNKD*.^{87,88,95} This gene encodes a protein that may suppress synaptic vesicle release by interacting with RIM1 and RIM2.¹⁰² Although *SLC2A1*, *PNKD*, and *PRRT2* harbor the majority of the causal mutations for the genetically defined cases of PxD,⁸⁷ a few patients have been reported to carry potentially causal mutations in other genes such as *KCNMA1*¹⁰³ and *PDHA1*.¹⁰⁴ Efforts to identify the mutations responsible for PxD in other families have not yet been successful.⁸⁸

Dogs can also develop PxD.¹⁰⁵ Published reports have provided clinical descriptions of primary canine PxD (cPxD) or cPxD-like diseases in several breeds including the Bichon Frise,¹⁰⁶ Border Terrier,¹⁰⁷ Cavalier King Charles Spaniel,¹⁰⁸ Chinook,¹⁰⁹ Doberman Pinscher,¹¹⁰ English Bulldog,¹¹¹ Scottish Terrier,¹¹² and Soft-Coated Wheaten Terrier (SCWT).¹¹³ Unlike the human primary PxD that are almost always dominant traits, the cPxD usually have a recessive mode of inheritance. The cPxD that occurs in the Cavalier King Charles Spaniel is typically

precipitated by exercise.¹⁰⁸ It is caused by a homozygous 16-kb microdeletion that includes the first three exons of *BCAN*^{114,115}, which encodes the extracellular-matrix protein brevican. The molecular genetic causes of the other cPxD have not yet been reported. We now present evidence that SCWT with cPxD have a deficiency in the biosynthesis of glycosylphosphatidyinositol (GPI) anchors due to a homozygous missense mutation in *PIGN*, the gene that encodes a GPI synthesis enzyme, GPI ethanolamine phosphate transferase-1.

Methods

Animals

Medical records and available videos of dyskinesia episodes were reviewed for 22 SCWT and 3 “Whoodles” (SCWT × Poodle crosses) with cPxD. The median age of onset of signs was 2.25 years. Males and females were affected equally. All dogs had normal neurologic exams between the episodes. Episode duration ranged from several minutes up to >4 h, and the frequency of episodes ranged from 1 every few days to >10/day. In six cases, stress, excitement, or loud noises were thought to precipitate some attacks, but a clear trigger was not apparent in most instances. No dogs had a history of ingestion of alcohol or caffeine. Typical episodes consisted of rapid flexion and extension of the hind limbs with varying degrees of truncal dystonia (Supplementary Video: https://static-content.springer.com/esm/art%3A10.1007%2Fs10048-016-0502-4/MediaObjects/10048_2016_502_MOESM3_ESM.wmv). Most frequently, the flexion alternated irregularly between limbs, but sometimes both hind limbs were

off the ground simultaneously (Fig. 1). In severe episodes, the front limbs were also affected. No consistent improvement in episodes was reported with anti-epileptic drugs or benzodiazepines. The signs typically worsened with age, and six dogs were euthanized within 2 years after onset of signs due to the increasing severity of the episodes. MRIs were obtained from five affected dogs, and postmortem exams were performed on four dogs, but no abnormalities were identified in any of the examined brains.

Molecular Genetics

DNA was collected from 25 cPxD-affected dogs. In addition, we used archived DNA samples from 665 cPxD-free SCWT, 388 Poodles, and 132 randomly selected dogs from other breeds to validate the identified candidate causal variant. Whole genome sequences were generated with DNA from two different cPxD-affected SCWT. PCR-based DNA libraries were used for the first whole genome sequence as previously described.¹¹⁶ PCR-free DNA libraries and slightly modified procedures¹¹⁷ were used for the second. Both data sets were deposited in the Sequence Read Archives (accession numbers SRX863100 and SRX863101). Previously described procedures were used for trimming adaptor sequences from the sequence reads, eliminating duplicate reads, error correction, and alignment to the CanFam3.1 canine reference genome sequence.^{52,116} Sequence variant information was uploaded to a custom PostgreSQL database, which also contained sequence variant information from the aligned whole genome sequences of normal canids or dogs with diseases other than cPxD. These other sequences served as controls for the current analysis. The University of Missouri

DNA Core Facility generated 43 of the control dog genome sequences; the rest were provided by individuals at other institutions as indicated in the “Acknowledgements”.

PCR primers 5'-GCTATACTAATATTTACCGTTC-3' and 5'-AAAATATAGTAAGTAATACACAA-3' were used to amplify a segment of genomic DNA containing candidate sequence variant *PIGN*:c.398C>T, identified from the whole genome sequences, so that the sequence variant could be verified by direct automated Sanger sequencing. An allelic discrimination assay⁸¹ was used to genotype DNA samples from individual dogs at this sequence variant. PCR primers for this assay were 5'-TGTGGGATTTGATTCTCTTATTAATG-3' and 5'-TCAATGACTCTTACCTTTGGCAAACATA-3'. The competing probe sequences were 5'-VIC-CCAGCTCCATGTGTACC-MBG-3' (reference C allele) and 5'-FAM-CCAGCTCCATATGTACC-MBG-3' (variant T allele).

PIGN-knockout HEK293 cells were generated and transfected as previously described¹¹⁸ with human wildtype or mutant (T133I, T133S, or T133V) *PIGN* complementary DNA (cDNA) cloned into pME, a strong SR α promoter-driven expression vector, or pTK, a medium TK promoter driven expression vector. These *PIGN* constructs had an HA epitope tag at the N-terminus. Transfection efficiencies were determined by the luciferase activity of cell lysates. After 3 days of incubation, restoration of the cell-surface expression of CD59 was evaluated by flow cytometry.¹¹⁸ Levels of expressed wild-type and mutant HA-tagged *PIGN* in pME vector-transfected cells were determined by western blotting using an anti-HA antibody.

Fig. 1. A 4-year-old, SCWT during a dyskinesia episode showing hyperflexion of the hind limbs that elevated both limbs off the ground simultaneously.

Results

Molecular genetics

Partial pedigree information was available for some of the affected dogs. Because no records indicated that the sires or dams of any of the affected dogs also exhibited cPxD, we assumed an autosomal recessive inheritance in our data analysis.

The first generated whole genome sequence from a cPxD-affected dog had a 21-fold average coverage of the reference genome and contained 6.9 million potential sequence variants (homozygous or heterozygous differences from the reference canine genome sequence). When this sequence variant information was first

added to a custom PostgreSQL database, it also contained sequence variant information from eight control canine WGSs. The SCWT sequence variants were filtered to identify only those that fit three criteria: (1) they were predicted to alter the amino acid sequence of the gene product (including those that alter exon-splicing signals), (2) they were homozygous in the cPxD-affected dog's whole genome sequence, and (3) they were absent from the control whole genome sequences. In the initial analysis, 418 sequence variants met these criteria. However, no plausible candidate sequence variants were identified from a review of the published biological functions and disease associations for the 302 genes that harbored these sequence variants.

As additional control whole genome sequences were added to the database, the number of homozygous coding variants unique to the affected SCWT decreased. Nonetheless, we were still unable to recognize a potential cPxD-causing sequence variant. Thus, we generated a whole genome sequence from another cPxD-affected dog. This sequence had 18-fold average reference genome coverage and contained 5.5 million potential sequence variants. By the time that the variants from the second cPxD-affected SCWT were included in our database, it contained variant data for 100 canid sequences that could be considered to be controls for the SCWT PxD. At this time, the whole genome sequence for the first case SCWT contained 230 variants in 162 different genes that met our criteria for candidate causality (Supplementary Table 1a). The whole genome sequence for the second SCWT PxD case had 65 candidate variants in 54 different genes (Supplementary Table 1b). The higher number of variants from the first case is attributable to the

many false positives among the filtered variants from the early PCR-amplified libraries. The only sequence variant common to both animals was *PIGN:c.398C>T*, which predicted a p.T133I substitution in PIGN, the gene product. Direct automated Sanger sequencing of PCR amplicons produced with primers spanning the *PIGN:c.398C>T* variant confirmed that both of the SCWT with cPxD were *PIGN:c.398T* homozygotes.

All 25 of our DNA samples from cPxD-affected dogs tested homozygous for the variant *PIGN:c.398T* allele. Twenty-two of these affected homozygotes were SCWT, and the other three were Whoodles. Whoodles are marketed as the product of F1 crosses between purebred SCWT and purebred Poodles. If this was indeed the origin of the three c.398T homozygous Whoodles, it would require that the *PIGN:c.398T* allele had been segregating in both breeds. Thus, we genotyped individual representatives from each breed at *PIGN:c.398C>T*. Fifteen of the 682 control SCWT were heterozygotes; the remaining 667 were homozygous for reference allele c.398C. All 388 of the genotyped Poodles were *PIGN:c.398C* homozygotes, as were all 132 randomly selected representatives of 92 other breeds. Although the *PIGN:c.398T* allele was not present in the Poodles that we genotyped, it could still be carried by other Poodles. Nonetheless, an alternative and more likely explanation for the origin of the three homozygous Whoodles is that they resulted from a Whoodle to SCWT backcross or from two Whoodle parents.

Flow cytometry

The biological activities of mutant *PIGN* isoforms were assessed by transiently transfecting corresponding cDNAs into *PIGN*-knockout HEK293 cells and measuring the restoration of GPI-anchored CD59 surface expression.^{118,119} Flow cytometry of cells which were transfected with a medium promoter-driven expression vector revealed comparable restoration of CD59 cell-surface expression by *PIGN*-knockout HEK293 cells 3 days after transient transfection with wild-type human *PIGN* cDNA or with recombinant human cDNAs designed to encode PIGN:p.T133S or PIGN:p.T133V isoforms. However, transient transfection with recombinant human cDNAs designed to encode PIGN:p.T133I restored only 60–70% of the cell-surface CD59 that was produced by the wild-type cDNA (Fig. 2a). The same T133I *PIGN* cDNA, when expressed with a strong promoter-driven vector pME, nearly fully restored CD59 (Fig. 2a). Western blotting revealed comparably expressed protein levels of T133I and wild-type PIGN (Fig. 2b). It is therefore concluded that the T133I mutation causes a reduction in specific activity rather than stability of PIGN.

Fig. 2. Functional analysis of *PIGN* mutants in *PIGN*-knockout HEK293 cells. **a.** Restoration of cell-surface expression of GPI-anchored protein CD59 on *PIGN* knockout HEK293 cells after transfection. HA-epitope-tagged, wild-type T133I, T133S, and T133V human *PIGN*:cDNAs were transfected with a medium promoter-driven pTK vector (top) or a strong promoter-driven pME vector (bottom). Three days later, CD59 levels were assessed by flow cytometry. **b.** Western blotting analysis of wild-type T133I, T133S, and T133V human *PIGN*. *PIGN*-knockout HEK293 cells that were transfected with HA-epitope-tagged, wild-type and mutant *PIGN* cDNAs in pME were analyzed 3 days later by western blotting using anti-HA antibody. GAPDH glyceraldehyde 3-phosphate dehydrogenase, a loading control.

Discussion

We studied a cPxD originally described in SCWT in 2004,¹¹³ which shared features of human PxD. All the affected dogs were normal on examination between episodes. The movements appeared to be involuntary and could not be disrupted. The dogs remained conscious during the episodes, and except for severe episodes, the dogs continued to walk, suggesting that the movements were semi-purposeful. Some of the movements in this cPxD were the sustained, abnormal postures of a dystonia.¹²⁰ The more rapid movements in cPxD are hyperkinetic movements, but they are difficult to place into one of the human categories of chorea, ballism, or athetosis.¹²¹ The various forms of human PxD can be subclassified as a PED, PKD, or PNKD.^{83-86,88,89} The owners of dogs with cPxD never mentioned exercise or fasting as triggers of dyskinesia episodes as occurs with PED in humans.¹²² Determining whether a sudden movement triggered the episodes as in PKD in humans⁸⁹ was difficult since the owners typically were not closely observing their dogs at the onset of the episodes. Compared to the durations and frequencies of episodes of dyskinesia in human PKD patients,⁸⁹ the episodes in dogs with *PIGN*-associated cPxD lasted longer (minutes to hours) and occurred less frequently (several/day to every few weeks), more like PNKD in humans.⁸⁸ Stress and excitement, which trigger episodes in most PNKD associated with *PNKD* in humans⁸⁸ were reported to trigger the episodes in some dogs, although in most cases, no trigger could be identified. While alcohol or caffeine ingestion are the most consistent triggers of human PNKD associated with *PNKD* mutations,⁸⁸ dogs rarely ingest alcohol and methylxanthines are toxic to

dogs,¹²³ so whether they would trigger episodes in dogs is unknown. PKD patients either require no treatment or respond to anti-epileptic drugs, and PNKD patients often respond to benzodiazepines,^{84,87-89} while SCWT with cPxD showed a poor response to these drugs. The median age of onset in dogs was 2.25 years, a young but fully mature adult dog, which would be older than the equivalent average age of onset in humans of PNKD associated with *PNKD* or PKD and more comparable to "atypical" PNKD not associated with *PNKD* mutations.^{88,89} Thus, even though there are differences, the dyskinesia episodes in *PIGN*-associated cPxD most closely resemble those of human PNKD patients.

To identify the molecular genetic cause of cPxD, we generated whole genome sequences for two affected dogs. The T allele of a *PIGN*:c.398C>T transition was the only rare, homozygous, predicted amino acid altering sequence variant that was found in both of these animals. All 25 of our c.398T allele homozygous DNA samples were from cPxD-affected dogs, whereas none of the 1053 control SCWT and Poodles and none of the 132 genotyped dogs from other breeds were c.398T homozygotes. This established a strong association between the clinical disease and T allele homozygosity. None of the 15 *PIGN*:c.398C/T heterozygous SCWT were known to have exhibited episodes of dyskinesia, which supports the inheritance of SCWT cPxD as an autosomal recessive trait.

PIGN is 1 of more than 26 genes that encode the polypeptides which contribute to the biosynthesis, protein attachment, and remodeling of GPI anchors. GPI anchors tether a diverse group of cell-surface proteins to the plasma membrane (Fig. 3a). Amide bonds covalently attach the cell-surface protein to an ethanolamine

phosphate at the outer end of the GPI anchor, while at the inner end, aliphatic chains intercalate into the plasma membranes and bind to hydrophobic lipid-raft components. A complex core glycolipid provides a covalent bridge between the two ends of the GPI anchor.^{119,124} During GPI assembly in the endoplasmic reticulum, ethanolamine phosphate side chains are added to mannosyl moieties in the core glycolipid. GPI ethanolamine phosphate transferase-1 (PIGN), the enzyme encoded by *PIGN*, facilitates the transfer of an ethanolamine phosphate from phosphatidylethanolamine to the innermost mannosyl moiety in a partially assembled core glycolipid intermediate (Fig. 3b).¹²⁵⁻¹²⁷ The existence of *PIGN*-associated deficiency diseases indicates that this ethanolamine phosphate side chain has an important biological function, probably related to its role in the recognition of GPI glycolipids by the transamidase complex that attaches proteins to the GPI anchors.¹²⁸ Flow cytometry has been used to demonstrate altered expression of these proteins with mutations of *PIGN* in humans.^{118,129-131}

Fig. 3. Structure and synthesis of GPI. **a.** the core structure of the GPI anchor is a phosphatidylinositol moiety, a glucosamine moiety, three mannoses, and a ethanolamine phosphate (EtNP) on the terminal mannose. The lipid tails of PI are embedded in lipid rafts in the plasma membrane, and cell-surface proteins are bound to the terminal EtNP. Another EtNP bound to the first mannose is found in all mammalian cells. **b.** *PIGN* adds the EtNP to the first mannose during the synthesis of GPI in the endoplasmic reticulum.¹¹⁹

The variant *PIGN*:c.398T allele is predicted to result in a missense mutation, *PIGN*:p.T133I, in the phosphodiesterase domain. Evidence that the *PIGN*:p.T133I substitution partially impairs biological function was obtained by transiently transfecting *PIGN*-knockout HEK293 cells with a human *PIGN* cDNA construct designed to encode the p.T133I substitution. As demonstrated by flow cytometry, the human *PIGN*:p.T133I cDNA was ~60% as effective as wild-type *PIGN* cDNA in restoring CD59 surface antigen to the *PIGN*-knockout cells. Available sequence information indicates that *PIGN* orthologs in at least 146 vertebrate species have a threonine codon at the position orthologous to codon 133 in the human and

canine *PIGN*; however, at least two vertebrate species, the pig and the cape elephant shrew, have a serine codon at this position (Supplemental table at: https://static-content.springer.com/esm/art%3A10.1007%2Fs10048-016-0502-4/MediaObjects/10048_2016_502_MOESM2_ESM.tif). This suggested that the hydroxyl moiety of threonine or serine might be required for normal biological function. To test this hypothesis, we transiently transfected *PIGN*-knockout HEK293 cells with recombinant human *PIGN* cDNA designed to encode either *PIGN*:p.T133S or *PIGN*:p.T133V. With both of these constructs, the restoration of CD59 surface antigen was similar to that produced by the wild-type *PIGN* cDNA. The apparently normal function of *PIGN* with a valine at position 133 indicates that hydroxyl moiety at this position is not a functional requirement. Nonetheless, the contrast in flow cytometry patterns obtained with the various cDNAs substantiates the significance of the 30–40% decrease in CD59 surface antigen restoration that was obtained with the cDNA containing the p.T133I variant.

N-ethyl-N-nitrosourea-derived mice with a homozygous truncating splice-site mutation in *Pign* die *in utero* with developmental deficiencies including holoprosencephaly.¹³² Similarly, a human fetus died with a diaphragmatic hernia and many other severe developmental abnormalities, apparently due to a homozygous nullifying splice-site mutation.¹³³ Individuals born with other homozygous or compound heterozygous *PIGN* mutations share a developmental deficiency syndrome known as multiple congenital anomalies-hypotonia-seizures syndrome-1 or MCAHS1 (OMIM #614,080). The current literature describes at least 17 MCAHS1 patients from 9 families carrying 13 different *PIGN*

mutations.^{118,129-131,134,135} These patients had neonatal hypotonia, severe developmental delays, congenital anomalies, visual impairment, hyporeflexia, tremors, and seizures. Some exhibited choreoathetosis and gait abnormalities, while others had structural brain abnormalities including delayed myelination, cortical atrophy, diffusion restriction in the globus pallidi and corticospinal tracts, and cerebellar atrophy or parenchymal loss in the vermis.^{118,131,135} The severity of clinical signs in MCAHS1 patients appears to correlate with the functional severity of the *PIGN* mutations. The most severe signs, which included multiple structural and functional developmental anomalies, occurred in a neonate with a frameshift mutation in trans with a multi-exon microdeletion. In contrast, a child with likely hypomorphic compound heterozygous missense *PIGN* mutations showed no dysmorphic features, moderate developmental delay, and a later onset of seizures and spastic quadriparesis.¹³⁵

The cPxD described in the current report is less severe than the human and murine *PIGN*-associated phenotypes. In contrast to the developmental delays and early mortality seen in humans^{118,131,135} and mice,¹³² respectively, with loss of *PIGN* function, the first episodes of dyskinesia occurred in young-adult dogs. The dogs appeared to be normal prior to the onset of the dyskinesia and between episodes. The marked differences in severity may be due to inherent differences between species. Alternatively, the differences in severity may occur because the canine mutation encodes a product which retains a relatively greater amount of residual functional activity. This second possibility is supported by the finding that transfection of *PIGN*-knockout HEK293 cells with human T133I *PIGN* cDNA in a

strong promoter-driven pME vector fully restored CD59 surface expression, whereas a recent report¹¹⁸ showed that transfection of *PIGN*-knockout HEK293 cells with the same strong promoter-driven vector and *PIGN* cDNA carrying the MCAHS1-associated S270P resulted in markedly reduced CD59 surface expression (see Fig. 2b in reference¹¹⁸). Thus, it seems possible that hypomorphic mutations of human *PIGN* could also cause PxD or other disease phenotypes that are less severe than those of MCAHS1. Similar variability in phenotype is seen in *SLC2A1* variants associated with PxD in humans.^{96,97,136}

In summary, we have described a paroxysmal dyskinesia in dogs that most closely resembles PNKD but differs from the known human PxDs; in that, it is autosomal recessively inherited. This disease is very likely caused by a hypomorphic missense mutation in *PIGN*, which encodes an enzyme in the biosynthetic pathway for GPI anchors. If so, it expands the phenotypes associated with altered GPI function and suggests candidate genes to investigate in human PxD that have not had a causal mutation identified. The spontaneous canine disease could serve as an animal model to investigate the pathogenesis of PxD and potential therapies. Nonetheless, we cannot rule out the possibility that a variant in tight linkage disequilibrium with the *PIGN*:c.398C>T transition is the true cause of the disease. Generation of a mouse model expressing the *PIGN*:c.398T variant that recapitulated the phenotype seen in dogs or the identification of other *PIGN* variants in dogs with cPxD would help confirm that the variant is the causal and permit further studies on its effect on GPI function and the difference in phenotype with the null mutations in mice and humans.

Supplemental Table 1a: Homozygous unique coding variants in first whole genome sequence produced with PCR-amplified libraries.

GENE	CFA	CHR_POS	REF_BASE	VARIANT	AA_CHANGE	COVERAGE
<i>ACCN4</i>	37	26025744	A	C	289D>A	3
<i>ACCN4</i>	37	26025758	A	C	294T>P	4
<i>ACOXL</i>	17	35270351	T	G	132V>G	3
<i>ACOXL</i>	17	35270356	C	G	134L>V	3
<i>ADAMTS15</i>	5	4458444	T	A	787K>M	6
<i>AHNAK</i>	18	54109648	G	A	3709V>I	3
<i>ANXA7</i>	4	23951430	C	G	49A>P	2
<i>ANXA7</i>	4	23951436	G	A	47P>S	2
<i>ANXA7</i>	4	23951442	T	C	45T>A	2
<i>ANXA7</i>	4	23951448	C	T	43G>R	2
<i>ARHGAP20</i>	5	22230860	C	delCCC	In-Frame	2
<i>ASPG</i>	8	71757312	T	G	129V>G	2
<i>ATP13A2</i>	2	81214960	T	C	1148F>S	5
<i>BAI2</i>	2	69360924	A	C	271N>T	3
<i>BAI2</i>	2	69360926	T	C	272S>P	3
<i>BEGAIN</i>	8	68796283	T	G	191D>A	8
<i>BRWD1</i>	31	34164606	C	A	970G>C	2
<i>C10H2orf55</i>	10	43842573	A	C	423T>P	7
<i>C14H7orf60</i>	14	52424229	G	A	151T>I	2
<i>C17H1orf56</i>	17	60135634	A	C	58K>Q	8
<i>C20H19orf29</i>	20	55815800	T	C	582S>P	5
<i>C30H15orf61</i>	30	31644657	A	C	114N>T	5
<i>C37H2orf47</i>	37	9138416	T	G	226C>G	2
<i>CADM2</i>	31	1965176	C	T	297E>K	8
<i>CAPZA2</i>	14	55809759	C	G	129R>G	4
<i>CCDC40</i>	9	1641190	G	C	338A>G	3
<i>CCNDBP1</i>	30	10054083	A	C	39E>A	6
<i>CD14</i>	2	35792097	C	A	156G>W	40
<i>CDK18</i>	38	1999372	A	C	448Q>H	2
<i>CEP135</i>	13	48248017	A	G	437D>G	5
<i>CNO</i>	3	58822876	T	G	156V>G	6
<i>CNO</i>	3	58822878	T	G	157Y>D	6
<i>CRBN</i>	20	14247670	G	A	142E>K	3
<i>CSNK2A2</i>	2	58398399	C	G	5P>A	2
<i>CSNK2A2</i>	2	58398400	C	G	5P>R	2
<i>CSNK2A2</i>	2	58398402	C	G	6P>A	2
<i>CSNK2A2</i>	2	58398403	C	G	6P>R	2
<i>CYP11B2</i>	13	36877031	A	C	239Y>S	3
<i>DCTN6</i>	16	34124786	G	T	97Q>K	4

GENE	CFA	CHR_POS	REF_BASE	VARIANT	AA_CHANGE	COVERAGE
<i>DCTN6</i>	16	34124789	A	G	96S>P	4
<i>DIRC2</i>	33	25910104	T	G	298S>A	2
<i>DLA-12</i>	12	933665	G	A	58D>N	3
<i>DLL1</i>	12	72261728	T	C	1204X>W	3
<i>DLL1</i>	12	72261743	A	G	1199M>T	3
<i>EBF1</i>	4	51381583	A	G	78Q>R	8
<i>EBF1</i>	4	51381586	G	C	79G>A	8
<i>EID1</i>	30	15109191	C	A	10L>M	2
<i>EID1</i>	30	15109274	C	G	37S>R	7
<i>ESD</i>	22	4569705	T	C	264F>L	3
<i>FAF1</i>	15	10425121	G	C	144W>S	5
<i>FAM120C</i>	39	45943538	T	G	7K>Q	2
<i>FAM120C</i>	39	45943539	T	G	6K>N	2
<i>FAM120C</i>	39	45943540	T	A	6K>I	2
<i>FAM120C</i>	39	45943541	T	A	6K>X	2
<i>FAM120C</i>	39	45943542	T	G	5E>D	2
<i>FAM120C</i>	39	45943543	T	C	5E>G	2
<i>FAM131A</i>	34	17222704	C	insG	FS	2
<i>FAM131A</i>	34	17222709	T	G	405L>R	2
<i>FAM98A</i>	17	26677147	G	C	236S>C	4
<i>FBRSL1</i>	26	536801	T	G	770D>A	9
<i>FFAR1</i>	1	117257822	T	G	39T>P	3
<i>FRYL</i>	13	44398730	T	C	664D>G	6
<i>FRYL</i>	13	44398733	T	C	663E>G	6
<i>FTH1</i>	11	39037568	A	G	14H>R	3
<i>GALNT13</i>	36	973660	T	C	294L>P	2
<i>GEM</i>	29	39025840	A	G	241L>P	2
<i>GLB1</i>	23	3769351	A	G	276D>G	6
<i>GPAA1</i>	13	37579195	C	G	394I>M	2
<i>GPAA1</i>	13	37579198	T	G	395H>Q	2
<i>GPAA1</i>	13	37579201	C	G	396F>L	2
<i>GPRASP1</i>	39	76315045	C	G	436R>G	7
<i>GPRASP1</i>	39	76315046	G	A	436R>Q	7
<i>GPRASP1</i>	39	76315047	G	insA	FS	7
<i>GPRASP1</i>	39	76315048	T	A	437W>R	7
<i>GPRASP1</i>	39	76315049	G	A	437W>X	7
<i>GPRASP1</i>	39	76315055	A	T	439D>V	7
<i>HDAC10</i>	10	17005973	T	C	735S>P	8
<i>HDAC10</i>	10	17005979	A	C	737T>P	8
<i>HMP19</i>	4	38493737	T	G	136I>L	10
<i>HOXC13</i>	27	1322988	A	G	128L>P	3

GENE	CFA	CHR_POS	REF_BASE	VARIANT	AA_CHANGE	COVERAGE
<i>HR</i>	25	35131009	C	insG	FS	6
<i>HR</i>	25	35131010	A	G	847Q>R	6
<i>HR</i>	25	35131013	A	G	848E>G	6
<i>IL17B</i>	4	59583768	G	C	115D>H	8
<i>IRF2BP1</i>	1	109740090	C	insG	FS	2
<i>ITGB3BP</i>	5	46682272	A	G	19N>D	2
<i>KCNA4</i>	21	50116932	A	C	192V>G	4
<i>KCNA4</i>	21	50116935	A	C	191V>G	4
<i>KCNF1</i>	17	7752774	C	G	42L>V	2
<i>KIAA1109</i>	19	17956232	T	A	1069E>V	5
<i>KIAA1109</i>	19	17956233	C	A	1069E>X	5
<i>KIF20B</i>	28	4621592	A	G	764T>A	5
<i>KIF26A</i>	8	71822968	T	C	72L>P	3
<i>LAMA4</i>	12	68574102	T	G	672H>P	5
<i>LOC100682952</i>	10	8635905	T	C	1M>V	3
<i>LOC100683298</i>	20	54432891	T	C	195S>P	5
<i>LOC100683298</i>	20	54432895	A	C	196N>T	5
<i>LOC100685794</i>	5	36098038	T	A	3M>K	2
<i>LOC100688783</i>	26	27216883	A	T	92T>S	9
<i>LOC100688783</i>	26	27216904	G	A	99E>K	9
<i>LOC100855692</i>	10	61863067	A	G	333I>T	2
<i>LOC100855864</i>	8	72736858	T	G	108S>A	9
<i>LOC100856304</i>	2	18383718	T	G	74D>A	2
<i>LOC100856304</i>	2	18383724	A	T	72L>H	2
<i>LOC100856304</i>	2	18383729	A	C	70F>L	2
<i>LOC100856351</i>	11	39043361	A	G	81C>R	2
<i>LOC477695</i>	27	35133108	C	T	142H>Y	2
<i>LOC487073</i>	2	1320994	T	G	864V>G	6
<i>LOC487543</i>	30	13354528	C	G	121A>G	2
<i>LOC487670</i>	30	39945445	C	G	535A>P	6
<i>LOC487815</i>	32	1475718	T	G	273Q>P	5
<i>LOC490390</i>	7	39229174	A	G	235E>G	8
<i>LOC490390</i>	7	39229176	C	G	236Q>E	8
<i>LOC607594</i>	25	24797574	A	G	51E>G	5
<i>LOC607594</i>	25	24797576	C	G	52R>G	5
<i>LOC610891</i>	38	23651499	T	G	896V>G	2
<i>LOC611902</i>	17	37499510	A	G	116N>D	2
<i>LOC611902</i>	17	37499511	A	G	116N>S	2
<i>LOC611902</i>	17	37499513	T	A	117L>I	2
<i>LONRF3</i>	39	91106869	A	G	125E>G	6
<i>LPHN1</i>	20	48380578	C	G	1445P>A	2
<i>LPHN1</i>	20	48380578	C	G	1445P>A	2

GENE	CFA	CHR_POS	REF_BASE	VARIANT	AA_CHANGE	COVERAGE
<i>LRPPRC</i>	10	46438325	T	G	6R>S	2
<i>LRRC27</i>	28	40298617	A	G	858E>G	6
<i>LRRC33</i>	33	29699324	C	G	682P>A	6
<i>LRRC45</i>	9	298900	T	G	365D>A	2
<i>MAPKAPK2</i>	7	5901211	A	C	241T>P	9
<i>MBIP</i>	8	14886709	T	C	322S>G	2
<i>MEAF6</i>	15	4947987	T	G	81V>G	6
<i>MRGPRG</i>	18	47136179	C	G	100R>P	10
<i>MRPL46</i>	3	51702661	T	G	184T>P	3
<i>MYO5A</i>	30	18052103	C	G	952R>P	2
<i>MYO5A</i>	30	18052106	G	C	951A>G	2
<i>NF1</i>	9	41505776	T	C	2212D>G	5
<i>NFKBIZ</i>	33	8125937	A	G	72K>R	2
<i>NFKBIZ</i>	33	8125951	T	C	77S>P	3
<i>NFKBIZ</i>	33	8125958	T	C	79F>S	3
<i>NFKBIZ</i>	33	8125965	G	C	81R>S	3
<i>NFKBIZ</i>	33	8125967	G	C	82G>A	3
<i>NID1</i>	4	3985012	C	G	720S>R	3
<i>NID1</i>	4	3985014	A	C	721H>P	3
<i>NUDT6</i>	19	17375074	A	G	131E>G	3
<i>PALM</i>	20	57855581	T	G	144T>P	7
<i>PARP14</i>	33	25782161	A	C	908E>A	8
<i>PIGN</i>	1	14705240	C	T	133T>I	16
<i>PKHD1L1</i>	13	10054619	T	G	3805C>W	11
<i>PKHD1L1</i>	13	10054621	C	G	3806A>G	11
<i>PLBD2</i>	26	10709971	C	A	134L>I	2
<i>PLBD2</i>	26	10709975	T	A	135F>Y	2
<i>PLBD2</i>	26	10709977	T	A	136L>M	2
<i>PLBD2</i>	26	10709981	C	A	137P>H	2
<i>PLEC</i>	13	37450827	T	G	4573Y>S	5
<i>PML</i>	30	37265646	C	T	500R>W	47
<i>PPM1E</i>	9	33350900	G	A	96G>D	3
<i>PRR12</i>	1	106921117	A	C	1554C>G	7
<i>PRSS33</i>	6	38320579	G	C	43R>G	6
<i>RAP1A</i>	17	63742031	C	A	25Q>H	2
<i>RAP1A</i>	17	63742032	T	G	25Q>P	2
<i>RBM10</i>	39	40762898	G	C	145G>R	2
<i>RBM10</i>	39	40762904	G	C	147G>R	2
<i>RBM10</i>	39	40762907	G	C	148G>R	2
<i>RBM10</i>	39	40762913	G	C	150G>R	2
<i>RFWD2</i>	7	23321045	A	G	114K>E	5
<i>RIN2</i>	24	3371828	T	G	387K>N	9

GENE	CFA	CHR_POS	REF_BASE	VARIANT	AA_CHANGE	COVERAGE
<i>RNASEH2B</i>	22	967992	G	insA	FS	6
<i>ROR2</i>	1	95278502	C	G	464A>G	3
<i>RRP1B</i>	31	37663897	G	insT	FS	2
<i>RSPH4A</i>	1	57074175	T	A	572D>E	3
<i>RXFP3</i>	4	73873384	A	C	243V>G	4
<i>S100PBP</i>	2	68499452	T	G	400T>P	3
<i>SCNN1B</i>	6	22462890	C	G	344A>P	5
<i>SCNN1B</i>	6	22462892	T	G	343N>T	5
<i>SEPT1</i>	6	17708699	A	G	117R>G	4
<i>SEPT1</i>	6	17708702	C	G	118P>A	4
<i>SLC12A3</i>	2	59437916	T	C	496K>E	12
<i>SLC25A46</i>	3	1718383	A	C	35L>R	4
<i>SLC25A46</i>	3	1718389	C	A	33G>V	4
<i>SLC25A46</i>	3	1718392	C	A	32G>V	4
<i>SLC25A46</i>	3	1718393	C	T	32G>R	4
<i>SLC29A3</i>	4	22098019	A	insC	FS	2
<i>SLC30A1</i>	7	9959075	C	G	104Q>H	2
<i>SLC35F5</i>	19	35270909	C	A	98N>K	5
<i>SLK</i>	28	16312240	T	C	146L>S	2
<i>SMAD6</i>	30	31013728	A	C	485T>P	5
<i>SMAD6</i>	30	31013731	T	C	486S>P	5
<i>SOX30</i>	4	52587962	A	G	203D>G	10
<i>SP7</i>	27	1886597	G	C	341E>Q	3
<i>SPACA1</i>	12	47450050	C	G	19L>V	13
<i>SPATA21</i>	2	81306486	G	C	433E>Q	4
<i>SPEN</i>	2	81687640	A	C	3227V>G	4
<i>SSC5D</i>	1	102196945	G	insA	FS	2
<i>SSC5D</i>	1	102196950	C	delC	FS	2
<i>STOML1</i>	30	37226705	C	T	127V>M	23
<i>SYNJ1</i>	31	27356222	G	A	412A>V	6
<i>TAF6L</i>	18	53899260	T	G	469T>P	5
<i>TAOK1</i>	9	43594263	A	C	175M>L	6
<i>TBXA2R</i>	20	55824993	C	G	195A>G	2
<i>TENC1</i>	27	2099162	C	G	1053E>D	2
<i>TGDS</i>	22	45386237	T	G	295R>S	3
<i>TGFA</i>	10	68910846	C	G	422V>L	5
<i>THEM4</i>	17	60925021	T	G	24H>P	3
<i>TJP3</i>	20	55722132	T	G	789D>A	4
<i>TMCC2</i>	38	1771057	G	C	443A>P	13
<i>TMEM151A</i>	18	51010388	G	C	145A>G	4
<i>TMPRSS9</i>	20	56680769	A	G	310L>P	2
<i>TOMM20</i>	4	4800927	C	T	136A>V	2

GENE	CFA	CHR_POS	REF_BASE	VARIANT	AA_CHANGE	COVERAGE
<i>TRIM23</i>	2	51353677	T	G	61T>P	3
<i>TRIOBP</i>	10	26884008	C	A	1584A>S	12
<i>TRIOBP</i>	10	26884010	C	G	1583G>A	12
<i>TRIOBP</i>	10	26884011	C	T	1583G>S	12
<i>TRIOBP</i>	10	26884028	G	A	1577P>L	2
<i>TRMT1</i>	20	49101235	C	G	29Q>E	11
<i>UCHL1</i>	3	71410964	A	T	3L>H	2
<i>ULK3</i>	30	37904557	A	G	23L>P	6
<i>USP25</i>	31	12652955	A	G	722R>G	3
<i>USP34</i>	10	61289706	C	G	2885A>P	5
<i>UTRN</i>	1	35876816	A	C	750Q>H	3
<i>VANGL2</i>	38	21828498	C	G	89G>R	10
<i>VANGL2</i>	38	21828499	C	G	88K>N	10
<i>VANGL2</i>	38	21828500	T	C	88K>R	10
<i>VIM</i>	2	19671672	C	T	457E>K	5
<i>WDR36</i>	3	1490475	T	C	75R>G	5
<i>WNT5B</i>	27	43670032	C	A	168Y>X	3
<i>YARS2</i>	27	16139787	G	A	97G>S	9
<i>YWHAE</i>	9	45512480	T	C	235M>V	3
<i>ZBTB4</i>	5	32388310	T	C	577I>V	2
<i>ZC3H6</i>	17	36646542	C	A	67H>N	2
<i>ZC3H6</i>	17	36646543	A	G	67H>R	2
<i>ZC3H6</i>	17	36646544	C	A	67H>Q	2

Supplemental Table 1b: Homozygous unique coding variants in second whole genome sequence produced with PCR-free libraries.

GENE	CFA	CHR_POS	REF_BASE	VARIANT	AA_CHANGE	COVERAGE
<i>ACTR5</i>	24	27154461	C	G	55A>G	3
<i>ACTR5</i>	24	27154463	T	G	56C>G	3
<i>ADAMTS19</i>	11	18273233	G	delG	FS	2
<i>APOB</i>	17	15888601	G	A	1560T>M	19
<i>ARHGEF26</i>	23	48250284	G	C	20R>P	3
<i>ARHGEF26</i>	23	48250289	G	A	22G>S	3
<i>ATP8B3</i>	20	57160284	G	C	1394R>S	10
<i>C18H11orf35</i>	18	25664137	C	G	739G>R	3
<i>C28H10orf82</i>	28	27224783	G	T	152Y>X	28
<i>C30H15orf39</i>	30	38175075	C	delCCGGCA	In-Frame	11
<i>C4H1orf96</i>	4	9878498	G	delGAGGCGGCG	In-Frame	8
<i>CABLES2</i>	24	46375466	T	G	138T>P	4
<i>CNNM3</i>	10	45154194	G	C	147P>R	2
<i>CSN1S1</i>	13	59430621	T	G	153D>E	26
<i>DFNB31</i>	11	68699676	C	delC	FS	2
<i>ECHDC2</i>	5	55972146	G	A	48R>Q	2
<i>FAM45A</i>	28	29346298	A	G	6S>G	2
<i>FAM45A</i>	28	29346301	G	insG	FS	2
<i>FAM73A</i>	6	69026458	G	A	527L>F	20
<i>FBXO45</i>	33	29635933	A	delAT	FS	2
<i>FBXO45</i>	33	29635937	T	insT	FS	2
<i>GPR83</i>	21	6536825	C	delCTT	In-Frame	2
<i>GTF3C1</i>	6	19168295	G	A	1639E>K	13
<i>JMJD8</i>	6	39891999	G	delGTGGCGCCGGTGCTGC	FS	4
<i>KCNQ3</i>	13	29062325	T	G	16N>T	3
<i>KIF12</i>	11	68368122	G	A	81A>V	13
<i>LATS1</i>	1	40446218	T	C	345I>V	11
<i>LEPREL2</i>	27	38194601	G	C	101S>W	3
<i>LOC100682747</i>	14	11647458	G	C	182E>Q	8
<i>LOC100682857</i>	16	22212954	A	T	235H>L	2
<i>LOC100682857</i>	16	22212957	T	delTCG	In-Frame	2
<i>LOC100684233</i>	12	36497100	T	delTTTTT	Splice	7
<i>LOC100684399</i>	35	765733	G	A	254G>R	5
<i>LOC100688053</i>	6	53880544	C	insT	FS	14
<i>LOC100855863</i>	38	22151133	C	G	589A>G	4
<i>LOC475279</i>	14	45015191	A	C	445V>G	4
<i>LOC484210</i>	1	98449334	G	delG	FS	4
<i>LOC484210</i>	1	98455777	C	G	1228A>P	3

GENE	CFA	CHR_POS	REF_BASE	VARIANT	AA_CHANGE	COVERAGE
LOC485980	24	47296889	C	delCC	FS	3
LOC489893	6	14726554	C	G	1164R>P	2
LOC610174	15	12776779	A	delA	FS	2
LOC610174	15	12776785	A	delAGACGG	In-Frame	2
LOC612378	6	57025306	C	G	147S>R	2
LOC612378	6	57025307	T	A	148S>T	2
LOR	17	61995003	T	G	229S>A	4
LOR	17	61995004	C	G	229S>C	4
LOR	17	61995153	T	G	279C>G	2
MAP3K15	39	15465777	T	C	29E>G	2
MAST3	20	44878332	A	C	1M>R	4
MIER2	20	58064321	C	A	245R>S	6
ODZ1	39	96111976	A	G	193L>P	3
PHLPP1	1	14234138	T	G	31E>A	2
PIGN	1	14705240	C	T	133T>I	16
PKD1L3	5	77754396	G	delG	FS	23
PLK5	20	57343915	A	G	11S>P	5
PRDM12	9	53284486	G	insGCG	In-Frame	5
PRR24	1	108705031	T	delT	FS	3
PRR24	1	108705036	T	C	20N>S	3
RAB12	7	74780877	G	A	87A>T	12
RIN3	8	1777689	C	T	319P>L	5
S1PR5	20	50644336	G	C	240A>P	2
SHISA2	25	13472951	A	delA	FS	2
SHISA2	25	13472952	A	T	382N>I	2
TMEM70	29	22480075	T	G	105C>G	6
TRIM44	18	32351165	G	delGGGCGG	In-Frame	6
TTC39A	15	9939623	A	C	285Y>S	4
ZMIZ2	99	264737	G	insC	FS	2

Chapter 3

Australian Cattle Dogs with Neuronal Ceroid Lipofuscinosis are Homozygous for a *CLN5* Nonsense mutation Previously Identified in Border Collies

Abstract

Young adult Australian Cattle Dogs have been diagnosed with neuronal ceroid lipofuscinosis (NCL), a fatal neurodegenerative disease. We characterized the Australian Cattle Dog form of NCL and determined its genetic cause. Tissues from 4 Australian Cattle Dogs with NCL-like clinical signs and buccal swabs from both parents of a fifth affected breed member, plus archived DNA samples from 712 individual dogs were used for this study. The tissue samples were examined by fluorescence, electron, and immunohistochemical microscopy. A whole-genome sequence was generated for 1 affected dog and a TaqMan allelic discrimination was used for genotyping. The accumulation of autofluorescent cytoplasmic storage material with characteristic ultrastructure in tissues from the 4 affected dogs supported the NCL diagnosis. The whole-genome sequence contained a homozygous nonsense mutation: *CLN5*:c.619C>T. All 4 DNA samples from clinically affected dogs tested homozygous for the variant allele. Both parents of the fifth affected dog were heterozygotes. Archived DNA samples from 346 Australian Cattle Dogs, 188 Border Collies, and 177 dogs of other breeds were homozygous for the reference allele. One archived Australian Cattle Dog sample was from a heterozygote. The homozygous *CLN5* nonsense mutation is almost

certainly causal because the same mutation had been previously reported to cause a similar form of NCL in Border Collies. The detection of this mutation allows testing to confirm the NCL diagnosis in this breed.

Introduction

The neuronal ceroid lipofuscinoses (NCLs) are fatal progressive neurodegenerative diseases characterized by the accumulation of autofluorescent lysosomal storage material within the brain, the retina, and other tissues. Mutations in 13 different genes have been found to cause various forms of NCL in humans.¹³⁷ This disease has been reported in a variety of wild and domestic animals including the dog.^{138,139} At least 10 DNA sequence variants from 8 different genes have been identified as the molecular genetic causes for NCLs in dogs (Table 1).^{51,52,117,140-148} Until recently, all of the previously identified NCL-causing sequence variants in dogs have been reported to occur within members of single breeds, although there are 2 examples of different NCL-causing alleles segregating independently in the same breed: mutations in *PPT1* and *TPP1* have been associated with different forms of NCL in Daschunds^{140,141} and mutations in *CLN6* and *CLN8* have been found in different NCLs of Australian Shepherds.^{52,144} Faller et al.⁵¹ were the first to report a previously identified NCL-causing mutation in a second dog breed when they described NCL in Chihuahua siblings that were homozygous for a single-base deletion and frameshift that had already been reported in a Chinese Crested Dog with NCL.¹¹⁷

Still to be identified are the molecular genetic causes of the previously reported NCLs in other breeds, such as the Australian Cattle Dog (also known as Blue Heeler),¹⁴⁹ Cocker Spaniel,¹⁵⁰ Dalmatian,¹⁵¹ Labrador Retriever,¹⁵² Miniature Schnauzer,¹⁵³ Polish Lowland Sheepdog,¹⁵⁴ and Saluki.¹⁵⁵ Mutations causing NCL are likely to be segregating in additional breeds for which the disease has not yet been reported. We had an opportunity to analyze biological samples from young adult Australian Cattle Dogs with NCL-like clinical signs. Herein, we describe the disease phenotype and report that its molecular genetic cause is identical to a mutation previously reported as the cause of NCL in Border Collies.¹⁴² Thus, ours is the second report that a NCL-causing mutation is present in a dog breed other than that of the original discovery.

Table 1. Breed distribution of canine NCL-associated mutations.

Disease	Gene	Mutation	Amino Acid Sequence	Affected Dog Breed
CLN1	<i>PPT1</i>	<i>PPT1:c.736_737insC</i>	p.F246Lfs*29	Dachshund ¹⁴⁰
CLN2	<i>TPP1</i>	<i>TPP1:c.325delC</i>	p.A108Pfs*6	Dachshund ¹⁴¹
CLN5	<i>CLN5</i>	<i>CLN5:c.619C>T</i>	p.Q207X	Border Collie ¹⁴²
CLN5	<i>CLN5</i>	<i>CLN5:c.934_935delAG</i>	CLN5:p.E312Vfs*6	Golden Retriever ¹⁴³
CLN6	<i>CLN6</i>	<i>CLN6:c.829T>C</i>	p.W277R	Australian Shepherd ¹⁴⁴
CLN7	<i>MFSD8</i>	<i>MFSD8c.843delT</i>	p.F282Lfs*13	Chinese Crested Dog, Chihuahua ¹¹⁷
CLN8	<i>CLN8</i>	<i>CLN8:c.491T>C</i>	p.L164P	English Setter ¹⁴⁵
CLN8	<i>CLN8</i>	<i>CLN8:c.585G>A</i>	p.W195X	Australian Shepherd ⁵²
CLN10	<i>CTSD</i>	<i>CTSD:c.597G>A</i>	p.M199I	American Bulldog ¹⁴⁶
CLN12	<i>ATP13A2</i>	<i>ATP13A2:c.1623delG</i>	p.P541fs*56	Tibetan Terrier ^{147,148}

Fig. 1. Photographs of four of the subject Australian Cattle Dogs. **(A)** Dog A from near St. Louis euthanized in 2014, **(B)** Dog B from Chicago euthanized in 2014. **(C)** Dog C from Seattle euthanized in 2007. **(D)** Dog E from Alabama euthanized in 2015.

Materials and Methods

Subject Australian Cattle Dogs

Five dogs were evaluated for this study. Although pedigree information was not available for all dogs, all were believed by their owners to be purebred Australian Cattle Dogs, and according to their veterinarians, all had physical characteristics that were typical of purebred Australian Cattle Dogs (Fig. 1). Australian Cattle Dog A, a 15-month-old spayed female (Fig. 1A), was referred to the Veterinary Specialty Services Neurology Department near St. Louis for an acute onset of

seizures and blindness in 2014. Approximately 2 weeks before presentation, the dog had experienced its first tonic-clonic seizure. The dog was reportedly completely blind in the postictal period. Partial return of vision occurred within 24 hours, but the dog never regained full vision. Over the next few days, the dog developed persistent focal seizures and “fly biting”. After the initial seizure, the dog circles constantly when not sedated. The dog had been evaluated by the local veterinarian who initiated the treatment with phenobarbital. There was no improvement with phenobarbital treatment and her care was transferred to the Neurology Department at Veterinary Specialty Services. The dog had been obtained from a breeder at a young age, but pedigree records and breeder contact information were lost, and it was not possible to locate closely related dogs. Although the owners originally described no visual deficits before the first seizure, they mentioned that the dog had once been quite athletic, but recently had not been successful playing Frisbee or fetch. The dog exhibited decreased coordination that included clumsiness and bumping into objects. Starting at approximately 1 year of age, the dog began to display signs of anxiety, particularly when encountering other dogs or unfamiliar people.

The physical examination was limited because dog A was extremely disoriented and hypersensitive to any auditory or tactile stimulation. With mild restraint, the dog would have severe myoclonic jerks. Cranial nerve examination identified a bilaterally absent menace reflex, but the remainder of the cranial nerve evaluation was normal. The dog was ambulatory with mild tetraparesis, proprioceptive ataxia, and delayed general proprioception in all 4 limbs. Dog A had intact segmental

reflexes. A CBC and serum biochemistry analyses were unremarkable. Magnetic resonance imaging identified diffuse brain atrophy indicated by widening of the cerebral cortical sulci, diffuse dilatation of the ventricular system, subjectively decreased size of the interthalamic adhesion, and increased cerebrospinal fluid (CSF) surrounding the folia of the cerebellum (Fig. 2). Because of the severity of the clinical signs, Dog A was euthanized the day after advanced diagnostic procedures were performed.

Australian Cattle Dog B, a neutered male (Fig. 1B), presented to the Chicago Veterinary Neurology and Neurosurgery Group in 2014 at approximately 18 months of age for evaluation of progressive neurologic changes and a recent severe seizure. The onset of signs occurred at approximately 12 months of age with periodic episodes of severe anxiety, particularly in response to loud noises. The severe anxiety persisted throughout the course of the disease. Over time, the dog's ability to recognize and respond to commands progressively decreased. As the disease progressed, other signs exhibited by Dog B included compulsive repetitive scanning of its surroundings, circling, loss of ability to navigate stairs, tremors, loss of coordination, visual impairment in both dim and bright light, periods of trance-like behavior, aggression, ataxia, and clumsiness including increased bumping into obstacles. At the time of the examination, the dog reacted aggressively to any attempts to control its head or body. Because of progressing neurological signs that did not abate with zonisamide and diazepam treatment, dog B was euthanized at approximately 20 months of age and a necropsy was

performed to collect tissues as described below. No pedigree information was available for this dog.

Fig. 2. T2-weighted MRI from Dog A in the sagittal (**A**) and transverse (**B**) planes. From Dog A, T1-weighted post-contrast MRI in the dorsal plane (**C**). CSF is white and brain parenchyma gray in the T2-weighted images. CSF is dark gray in the T1-weighted image. Diffuse brain atrophy is indicated by atrophy of the interthalamic adhesion (black arrow in A) dilatation of the 3rd (green dashed arrow in A) 4th (4-point star in A), and lateral ventricles (5-point stars in B and C), widening of the sulci of the cerebral cortex (solid yellow arrow in B), and increased CSF surrounding the folia of the cerebellum (yellow dashed arrow in A).

Australian Cattle Dog C (Fig. 1C) had been tentatively diagnosed with NCL in Pullman, Washington, in 2007. A DNA sample was received at the University of Missouri at that time and archived. The owners reported that during the 6 months before presentation, Dog C had begun showing abnormal behavior, including apparent incoordination, trance-like episodes, increasing fear of other dogs, and aggression towards the owners. At examination Dog C was disoriented, was overreactive to normal auditory and tactile stimuli, and had an intermittent left head tilt. Static knuckling was delayed in all 4 limbs, but no paresis was noted during observation of the gait. Tetra-ataxia also was noted, especially during pivoting. The menace response was absent bilaterally, but pupillary light reflexes were normal. Cerebrospinal fluid analysis disclosed a normal protein concentration (12mg/dL; reference range, <25 mg/dL) and a normal nucleated cell count (1/ μ L; reference range, <5/ μ L) with normal cell cytology. A 2-week course of prednisone was prescribed (1mg/kg PO q12h for 1 week, then 0.5 mg/kg PO q12h for 1 week) while awaiting additional testing (urine metabolic scree, CSF culture, serum titers for *Toxoplasma* and *Neospora*). Clinical signs were not altered by prednisone treatment. Results of the urine metabolic screen were normal, CSF culture was negative for bacteria at 3 days, and serum IgG and IgM titers for *Toxoplasma* and *Neospora* were negative. Because of the progression of its neurologic signs, the dog was euthanized at approximately 26 months of age.

A fourth Australian Cattle Dog (Dog D) presented at the Animal Medical Center in New York City in 2015 at the age of approximately 24 months after a 6-month history of progressive behavior changes, including aggression toward people and

other dogs, ataxia, anxiety, lethargy, and blindness. A neurologic examination identified the bilateral absence of the menace reflex and severely decreased nasal sensation. Pupillary light and palpebral reflexes were normal. The owners elected to have the dog euthanized immediately after the neurologic examination. Magnetic resonance imaging performed immediately after euthanasia showed diffuse symmetrical brain atrophy.

Australian Cattle dog E (Fig. 1D), a spayed female from Alabama, was euthanized in 2015 at approximately 27 months of age after having exhibited neurologic signs similar to the other 4 affected Australian Cattle dogs. Starting at about 6 months of age, the dog began to occasionally bump into obstacles, but continued to engage in vision-dependent activities, such as retrieving balls, without difficulty. Several months later, the dog began to exhibit signs of anxiety that became progressively more pronounced; it would become particularly agitated in response to loud noises. At approximately 2 years of age, the dog began to suffer from seizures and associated trance-like behavior, lost interest in activities it previously had enthusiastically engaged in, would pace compulsively, and began to suffer from progressive vision and coordination loss. Visual impairment was apparent in both dim and bright light. Upon neurologic examination at approximately 26 months of age, both direct and indirect pupillary responses could be elicited despite blindness. However, the dog did not exhibit a menace response on either side and physiologic nystagmus was observed in both eyes. Magnetic resonance imaging of the brain disclosed generalized brain atrophy, and enlarged ventricles. Cerebrospinal fluid analysis did not identify any abnormalities. Shortly after the

neurologic examination, the dog's seizures became more frequent and the dog was euthanized. No tissue or blood samples were saved, but buccal swab samples were collected from both parents.

Light and Electron Microscopic Procedures

Cerebral cortical (parietal lobe) cerebellar and retinal samples were collected at necropsy from 4 of the affected Australian Cattle Dogs and examined by fluorescence and electron microscopy. The eyes from dogs A and B were enucleated and the corneas removed immediately. One eye from each dog was placed in a fixative consisting of 3.5% formaldehyde, 0.05% glutaraldehyde, 120 mM sodium cacodylate, 1 mM CaCl_2 , and pH 7.4 (immune fix), and the other eye was placed in 2.5% glutaraldehyde, 100 mM sodium cacodylate, and pH 7.4 (EM fix). Slices of the cerebral cortex and cerebellum each were placed in the same fixatives. Before further processing, the eyecups were dissected to obtain regions from the posterior poles adjacent to the optic nerve heads and these regions were used for examination. All samples were incubated in these fixatives at room temperature until being further processed for microscopic examination. The same tissues were collected post-mortem from dogs C and D in a similar manner except that they initially were placed in 10% buffered formalin for shipment. In addition, a sample of heart ventricle wall was collected from dog C in the same fixative. Upon receipt at the University of Missouri, the tissue samples were washed briefly in 170 mM sodium cacodylate, pH 7.4 and then transferred to the same fixatives as used for the other dogs. These tissues were examined by previously described

histopathologic, immunohistochemical, fluorescent microscopy, and electron microscopic procedures.¹⁴³ Among these procedures was immunohistochemical staining of the cerebellar cortex and closely associated medulla were dissected from the brains fixed in immune fix (in some cases preceded by fixation in formalin). These tissues were embedded in paraffin and sectioned, the sections were immunostained for GFAP.

Molecular Genetic Analysis

EDTA-anticoagulated blood from affected Australian Cattle Dogs A, B, C and D described above served as a source of DNA. Buccal swab samples from the sire and dam of affected Australian Cattle Dog E were collected on commercial cards^a. Previously described procedures were used to isolate DNA from the blood¹¹⁷ and cards.¹⁵⁶ In addition, DNA samples from 347 other Australian Cattle Dogs, 188 Border Collies, and 177 dogs representing 88 other breeds also were used in the study. These samples were randomly selected from among the samples in the University of Missouri Animal DNA repository.

The DNA from Australian Cattle Dog A was submitted to the University of Missouri DNA Core facility for the preparation of 2 PCR-free libraries (fragment sizes of approximately 350 bp and 550 bp) with a commercial kit^b and for 2x100 paired-end sequencing in 2 flow-cell lanes in a massively parallel DNA sequencer.^c The adaptor sequences were trimmed with custom Perl scripts and the reads were error corrected using a previously described data analysis pipeline.¹¹⁷ These reads were deposited in the Sequence Read Archive (accession SRS834022) and

aligned to the canine reference genome sequence (CanFam 3.1) using the default parameters of the bwa algorithm in BWA 0.7.12 software.¹⁵⁷ Samtools 1.2 software was used to convert tab-delimited text files that contained sequence alignment data (aligned SAM file types) to binary versions (BAM file types), merge the libraries, and sort and index the files by genomic coordinates.¹⁵⁸ The Platypus 0.8.1 variant caller software¹⁵⁹ was used to generate individual variant call format (VCF) files using the default parameters. Commercial software^d was used to annotate variants and to compare them with similarly generated and annotated VCF files from 43 other control dogs with different genetic diseases. The same software^d was also used to filter the variants to identify those with the following 2 characteristics: (1) they were located within canine orthologs of the 13 genes associated with NCL in humans,¹³⁷ and, (2) they were predicted to alter the primary structure of the gene product.

The DNA samples from the 4 affected Australian Cattle Dogs were genotyped by direct automated Sanger sequencing of PCR amplicons produced with oligonucleotide primers 5'-TTAACCAAATGGCAAAGTGGG-3' and 5'-TTCTTGAAGTCTGCTCCAAGT-3' which span a previously described casual *CLN5*:c.619C>T transition.¹⁴² A TaqMan allelic discrimination assay⁸¹ was used to genotype both parents of Australian Cattle Dog E and the archived DNA samples. The sequences of the PCR primers for the allelic discrimination assay were 5'-GCGGGACAATGAAACAGGAATTTAT-3' and 5'-TGTCTCAGCCCCCTTTGTTG-3'. The competing probes were 5'-CTGGCTTGAACAGTCC-MGB-3' (reference allele) and 5'-CTGGCTTAAACAGTCC-MGB-3' (variant allele).

Results

Light and Electron Microscopy

All cerebella, cerebral cortical and retinal samples from the affected Australian Cattle Dogs exhibited abnormal accumulations of autofluorescent storage material with the fluorescence spectral characteristics typical of the NCLs (Fig. 3A-D). The cardiac muscle of a heart ventricle also was found to harbor substantial amounts of autofluorescent inclusions within the muscle fibers in the 1 affected dog from which the heart was examined (Fig. 3E).

Fig. 3. Fluorescence micrographs of cryostat sections the cerebellum (**A** and **B**), cerebral cortex (**C**), retina (**D**) from Dog A, and heart ventricular wall (**E**) from Dog C. In the cerebellum, storage body accumulation was most abundant in the Purkinje cell layer (pc) with some but not all of the material within cells that could be identified as Purkinje cells (arrows in **A** and **B**). There were lesser accumulations of this material in the molecular (m) and granular (g) layers. In the cerebral cortex (**B**), the storage material was widely distributed throughout most of the tissue. In the retina (**C**), the most prominent accumulations of autofluorescent material were in the ganglion cells (gc) and along the outer limiting membrane (olm). The retinal pigment epithelium (rpe) also contained substantial amounts of material with similar fluorescence properties, but since the accumulation of such material occurs during normal aging, the presence of this material in the rpe is not diagnostic for NCL. Clusters of autofluorescent inclusions were present in the heart muscle fibers sections in longitudinal orientation (**E**).

The ultrastructural appearances of the storage material in brain and retina were essentially the same for dog A and B from which EM-fixed samples were obtained.

The contents of the storage bodies had a primarily membranous appearance in cerebella Purkinje cells, cerebral cortical neurons, and retinal ganglion cells (Fig. 4). In all 3 cell types, the inclusions were membrane bound and consisted of

aggregated clusters of membrane-like structures, but the arrangement of this membrane-like material varied among cell types. The primary storage components in the Purkinje cells had short linear and vesicular appearances (Fig. 4A). In the cerebral cortical neurons and retinal ganglion cells, storage bodies consisted almost exclusively of clusters of parallel stacks of relatively linear profiles (Fig. 4B-D). The cerebral cortex contained storage bodies with scattered, extremely electron-dense structures (Fig. 4B). The degree of compaction of the membrane-like structure in the retinal ganglion cell inclusions was variable (Fig 4C and D). The storage bodies along the outer limiting membrane of the retina were located primarily within the photoreceptor cells just interior to the junctions between these cells and adjacent Mueller cells (Fig. 4E and F). These storage bodies contained either aggregates of small vesicles (Fig. 4E) or membrane-like material in a variety of configurations (Fig. 4F). The membrane-like components were randomly distributed within primarily granular material, unlike the clustered distributions found in the other cell types. The only cardiac tissue received from an affected dog was fixed in formalin, which did not permit good ultrastructural preservation of the storage material.

Fig. 4. Electron micrographs of the disease-related storage bodies from a cerebellar Purkinje cell (A), a cerebral cortical neuron (B), retinal ganglion cells (C and D), and retinal photoreceptor cells (E and F) of NCL-affected Australian Cattle Dog D. The storage bodies (s) in the photoreceptor cells are primarily located just internal to the outer limiting membrane. The contents of storage bodies consisted of both aggregates of small vesicles (E) or of membrane-like structures in a variety of configurations (F).

The NCLs, like many other lysosomal storage diseases, are characterized by astrogliosis, as indicated by increased amounts of glial fibrillary acid protein (GFAP) in astrocytes.^{52,117,143,160} Examination of the cerebellar cortex from Australian Cattle Dog A by immunohistochemistry disclosed very high numbers of cells in both the cerebellar medulla and the granule cell layer that stained strongly with an anti-GFAP antibody (Fig. 5A and C). By comparison, little GFAP immunostaining was observed in either of these cerebellar areas in a normal healthy dog of similar age (Fig. 5B and D).

Fig. 5. Light micrographs of sections of the cerebellar medulla (**A** and **B**) and granule cell layer (**B** and **D**). All sections were immunostained for GFAP. GFAP staining is a reddish brown color. All photomicrographs are from the cerebellum. Micrographs (**A**) and (**C**) are from the affected Australian Cattle Dog A; micrographs (**B**) and (**D**) are from a normal healthy Beagle of similar age. Bar in (**D**) indicates magnification of all 4 micrographs. GFAP-positive cells with the typical profiles of reactive astrocytes were abundant in both the granule cell layer and medulla of affected dog, but were not present in either of these areas of the cerebellar cortex from the normal dog.

Molecular Genetics

We used massively parallel sequencing technology to generate a whole-genome sequence from NCL-affected Australian Cattle Dog A and aligned the sequence reads to the canine genome reference sequence (CanFam3.1). The average depth of coverage of the aligned sequence was 28-fold. The 6.2 million potential sequence variants detected in this alignment were filtered to retain only those that were predicted to alter the amino acid sequences of any of the canine orthologs of the 13 genes known to have harbored mutations in human NCL patients.¹³⁷ As shown in Table 2, only 5 sequence variants survived this filtration process. Four were missense mutations that occurred in heterozygous state. These 4 variants were also present in from 5 to 13 of the 43 control whole-genome sequences from dogs with no signs of NCL and, thus, unlikely to have been responsible for Australian Cattle Dog A's NCL. In contrast, a *C* to *T* transition at position 30,574,637 bp on CFA22 was homozygous in Australian Cattle Dog A and produced a premature stop codon that was predicted to severely truncate the encoded CLN5. This variant was almost certainly causal because it previously had been reported to be responsible for NCL in Border Collies.¹⁴² The homozygous *CLN5:c.619C>T* transition, detected in the whole-genome sequence from Australian Cattle Dog A, was confirmed by automated Sanger sequencing. In addition, automated Sanger sequencing showed that Australian Cattle Dog B, C and D also were homozygous for the *CLN5:c.619T* allele.

Table 2. Variants in NCL-associated genes in WGS from affected Australian Cattle Dog A.

Chromosomal Coordinates	Gene	Nucleotide Change	Predicted Amino Acid Change	Zygosity	Comments
6:18257856	<i>CLN3</i>	<i>c.209A>G</i>	p.Glu70Gly	Heterozygous	Variant in 10 other WGSs
18:46011691	<i>CTSD</i>	<i>c.1168T>C</i>	p.Cys390Arg	Heterozygous	Variant in 10 other WGSs
18:46011691	<i>CTSD</i>	<i>c.761A>C</i>	p.Lys254Thr	Heterozygous	Variant in 13 other WGSs
21:2992271	<i>TPP1</i>	<i>c.1280C>T</i>	p.Ala427Val	Heterozygous	Variant in 5 other WGSs
22:30574637	<i>CLN5</i>	<i>c.619C>T</i>	p.Gln207*	Homozygous	Unique to affected dog WGS

A TaqMan allelic discrimination assay⁸¹ was used to determine the *CLN5*:c.619 genotypes for another 714 dogs, including both parents of the affected Australian Cattle Dog E. These 2 parents and 1 other Australian Cattle Dog represented in our DNA collection since 2014, were *CLN5*:c.619C/*T* heterozygotes. The variant c.619T allele was not detected in archived DNA samples from 346 other Australian Cattle Dogs previously obtained for a variety of reasons unrelated to NCL. In addition, all 188 randomly selected Border Collie DNA samples and all 177 randomly selected samples from representatives of 88 other breeds were homozygous for the reference c.619C allele.

Discussion

We used fluorescence and electron microscopy to confirm the diagnosis of NCL in 4 apparently unrelated Australian Cattle Dogs that had exhibited neurologic signs consistent with this disorder. Previously, NCL had been diagnosed in 2 pairs of Australian Cattle Dog littermates over 35 years ago.^{149,161,162} Both of the previously reported litters were from the United States, but there was no known familial relationship between them.¹⁶¹ Both sexes were affected. One litter included an affected pair and 7 clinical normal littermates.¹⁴⁹ The clinical status of the littermates to the other affected pair was not reported, but it was reported that their parents were normal.¹⁶¹ These observations are consistent with autosomal recessive mode of inheritance. The earlier-reported NCL-affected Australian Cattle Dogs and those that we studied had brains with similar distributions of autofluorescent storage bodies with similar ultrastructural appearance. In addition, the ages at onset of euthanasia of these earlier-reported Australian Cattle dogs with NCL are similar to those of the NCL-affected Australian Cattle Dogs reported here (Table 3). Thus, it is likely that a single founding mutation was responsible for both the earlier-reported Australian Cattle Dog NCL and for the affected Australian Cattle dogs reported here. Nonetheless, biological samples from the earlier-reported dogs were not available for testing and the existence of NCL phenocopies within the Australian Cattle Dog breed cannot be excluded.

Whole-genome sequencing has proven to be an efficient strategy for the identification of the mutations responsible for NCL in dogs.^{51,52,117,143} Thus, in search of the molecular cause of NCL in Australian Cattle Dogs, we generated a

whole-genome sequence with DNA from affected Australian Cattle Dog A and identified a homozygous nonsense mutation, *CLN5*:c.619C>T, that had already been reported to cause NCL in Border Collies.¹⁴² The NCL-affected Australian Cattle Dogs, B, C and D also were homozygous for the variant c.619T allele, whereas there were no c.619T homozygotes among 349 other Australian Cattle Dogs not known to have exhibited clinical signs of NCL. Three of those 349 Australian Cattle Dogs were c.619C/T heterozygotes including both parents of Australian Cattle Dog E. The similarities between the clinical history of Dog E and the clinical histories of the other 4 NCL-affected Australian Cattle Dogs, and the fact that both of Dog's E parents carried the rare c.619T allele strongly imply that Dog E was a c.619T homozygote. The association of NCL in 2 dog breeds with the same rare truncating mutation in a candidate gene strongly indicates that the mutation is causal for NCL in both Australian Cattle Dog and Border Collies.

Although there are anecdotal accounts of “dog show” Border Collies with NCL-like signs in Australia and New Zealand in the 1970s,¹⁶³ the first published report of NCL in Border Collies appeared in 1988.¹⁶⁴ This report described the disease in 3 male and 2 female Border Collies. Three years later, the clinical signs and laboratory findings for these 5 dogs and 18 other Border Collies were summarized.¹⁶⁵ As shown in Table 3, the age at disease onset and the disease duration in these Border Collies from Australia closely resembled that of the affected Australian Cattle Dogs, including those shown here to be *CLN5*:c.619C>T homozygotes. Subsequently, a Border Collie from the United States was reported to have NCL with similar clinical and laboratory findings but a later age at onset.¹⁶⁶

Table 3. Comparison of ages at onset and death or euthanasia for Australian Cattle Dogs, Border Collies, and Golden Retrievers with NCL.

Breed	Number of dogs	Causal Mutation	Location	Age at Onset	Age at Death or Euthanasia	Reference
Australian Cattle Dog	4	<i>CLN5:c.619T</i>	U.S.A.	12 to 19 months	15 to 26 months	Current report
Australian Cattle Dog	4	Unknown	U.S.A.	12 to 14 months	18 to 26 months	^{149,162}
Border Collie	23	Unknown	Australia	16 to 23 months	18 to 29 months	¹⁶⁵
Border Collie	1	Unknown	U.S.A.	27 months	29 months	¹⁶⁶
Border Collie	27	<i>CLN5:c.619T</i>	Japan	15 to 20 months	23 to 32 months	¹⁶⁷
Golden Retriever	4	<i>CLN5:c.934_935delAG</i>	U.S.A.	13 to 17 months	30 to 34 months	¹⁴³

In 2005, linkage analysis with DNA markers from candidate regions placed the Border Collie NCL locus on a *CLN5*-containing segment of canine chromosome 22 and the *CLN5:c.619C>T* transition was identified as the causal mutation.¹⁴² Since then, it has been possible to use DNA tests to identify c.619T homozygotes and to investigate the Border Collie NCL with genetically defined cohorts of NCL-affected dogs. Two reports described *CLN5:c.619T* homozygous Border Collies from Japan.^{167,168} The clinical laboratory findings for 27 Border Collies¹⁶⁷ with genetically defined NCL were similar to those from earlier reports of affected Border Collies and Australian Cattle Dogs and to those of the 5 Australian Cattle Dogs in our report. The ages at onset and death also were similar in each group of affected dogs (Table 3). The Japanese investigators found a *CLN5:c.619T* allele frequency >0.3 in dogs from certain kennels that unknowingly included asymptomatic heterozygotes in their breeding stock¹⁶⁷ and estimated an overall

CLN5:c.619T allele frequency of 0.04, based on random survey of over 400 Japanese Border Collies.¹⁶⁸ The mutant T allele appears to be less common in North America because 188 Border Collie samples from our collection all were *CLN5*:c.619C allele homozygotes. Although possible, it is very unlikely that the *CLN5*:c.619C>T transition arose independently in Border Collies and Australian Cattle Dogs. Both breeds are of similar size and both breed are used for herding. Some kennels produce puppies from both dog breeds. Thus, there is the potential for occasional planned or inadvertent exchange of genetic material between breeds. Published reports indicate that the disease has been present in both breeds for many generations,^{149,164} but it is not possible from the available evidence to identify the breed of origin. Regardless of the origin, a DNA test for the mutation can be used to aid in the diagnosis of NCL.

A different *CLN5* mutation, *CLN5*:c.934_935delAG, was found in the homozygous state in a Golden Retriever with NCL.¹⁴³ This mutation produces a reading frameshift and is predicted to encode a truncated gene product: *CLN5*:pE312Vfs*6. As shown in Table 3, the disease characteristics of the affected Golden Retrievers are similar to those of the affected Australian Cattle Dogs and Border Collies.

Mutations in human *CLN5* were first reported to cause NCL in 1998.¹⁶⁹ Since then, at least 36 potentially causal *CLN5* sequence variants have been found in human NCL patients (<http://www.ucl.ac.uk/ncl/cln5.shtml>). For most *CLN5* patients, the onset of clinical signs occurred between 4 and 7 years of age,^{170,171} but some presumably hypomorphic mutations have produced much later disease onsets.¹⁷²

Although CLN5 mutations have been recognized as causes of NCL for over 16 years, the biological functions of the gene product and the mechanism leading to the neurodegeneration remain unknown.¹⁷³ Ovine and murine Cln5-deficient animal models share disease features with human *CLN5* patients^{174,175} and have been used to investigate the underlying disease pathways.¹⁷⁶⁻¹⁷⁸ The ovine model is involved in ongoing and planned experiments to evaluate potential therapies for human CLN5 patients.¹⁷⁹ Several canine models have been used to develop therapies for lysosomal storage diseases,¹⁸⁰ and dogs have obvious advantages over sheep in urban research settings. A TPP1-deficient canine NCL model¹⁴¹ has proven useful in the evaluation of therapies for the human CLN2 form of NCL including an enzyme replacement therapy that is currently in clinical trials in human patients (<https://clinicaltrials.gov/ct2/show/NCT01907087>).¹⁸¹⁻¹⁸³ Dogs with CLN5 deficiency have the potential for similar use.

Footnotes

¹Whatman FTA Elute card, catalog number WB120411, Fisher Scientific, Chicago IL

²TruSeq DNA PCR-Free Sample Preparation Kit, Illumina, San Diego, CA

³HiSeq 2500 System, Illumina, San Diego, CA

⁴SNP & Variation Suite v8.x, Golden Helix, Inc., Bozeman, MT

Chapter 4

A homozygous *PPT1* splice donor mutation in a Cane Corso Dog with neuronal ceroid lipofuscinosis

Abstract

A 10-month-old spayed female Cane Corso dog was evaluated after a 2-month history of progressive blindness, ataxia and lethargy. Neurologic examination abnormalities indicated a multifocal lesion with primarily cerebral and cerebellar signs. Clinical worsening resulted in humane euthanasia. On necropsy there was marked gliosis throughout white matter tracts of the cerebrum, most prominently in the corpus callosum with moderate numbers of swollen axons and occasional spheroids. In the cerebral cortex and midbrain most neurons contained large amounts of autofluorescent storage material in the perinuclear area of the cells. Cerebellar storage material was present in the Purkinje cells, granular cell layer, and perinuclear regions of neurons in the deep nuclei. Neuronal ceroid lipofuscinosis (NCL) was diagnosed. Whole genome sequencing identified a *PPT1*c.124+1G>A splice donor mutation. This non-reference assembly allele was homozygous in the affected dog, has not previously been reported in dbSNP, and was absent from the whole genome sequences of 45 control canines and 31 unaffected Cane Corsos. Our findings indicate a novel mutation causing the CLN1 form of NCL in a previously unreported dog breed. A canine model for CLN1 disease could provide an opportunity for therapeutic advancement, benefiting both humans and canines with this disorder.

Case Report

A 10-month-old, 29 kg (63 lb), spayed female Cane Corso dog was referred to the University of Wisconsin, School of Veterinary Medicine with a 2-month history of progressive blindness, ataxia and lethargy. The dog was acquired by a family from a breeder at 8 weeks of age and was the only dog in the household. Both parents of the dog were healthy at the time this case presented. We were unable to obtain information on the health status of any littermates. Approximately 2 months prior to presentation, the dog had difficulty navigating in low light. Over the next 2 months, the visual impairment progressed to apparent blindness. Progressive ataxia was noted starting approximately 1 month prior to presentation, prompting referral.

On initial evaluation at the University of Wisconsin, the dog's physical examination was within normal limits. Abnormalities identified during a complete neurologic examination included a mild right head tilt, a positional right multifocal, most apparent in the prosencephalon and cerebellum. The preliminary differential diagnoses were meningoencephalitis, hydrocephalus, abiotrophy, neoplasia or lysosomal storage disorder.

The recommended diagnostic tests included a complete blood count, blood chemistry, urinalysis, urine metabolic screen for inborn ventral strabismus, an absent menace response bilaterally with intact pupillary light reflexes elicited by bright light stimuli, an absent paw replacement test of the left pelvic limb, and a moderate vestibular ataxia with truncal sway and hypermetria of all limbs. The

neuroanatomic lesion localization was considered errors of metabolism, infectious disease testing, thoracic radiographs, brain magnetic resonance imaging and cerebrospinal fluid analysis. All testing was declined except the urine metabolic test and infectious disease testing. Serum distemper by RT-PCR, serum *Neospora caninum* by IFA, the *Cryptococcus neoformans* antigen test and *Blastomycosis* urine antigen were all negative. The urine metabolic screen^a showed a slightly positive mucopolysaccharidosis spot test, which was attributed to young age, and glutamine and taurine levels were slightly above the reference range. The dog was discharged with instructions to administer prednisone (0.5 mg/kg per os q12h). Because the neurological condition continued to deteriorate, euthanasia was elected 5 days after discharge.

A complete necropsy was performed. Abnormal findings were limited to the central nervous system. The brain was bilaterally symmetrical and the cerebellar size was proportionate to the cerebrum, with no evidence of cerebellar herniation or ventricular dilatation. Histologic abnormalities were found in the cerebrum and cerebellum. In the cerebrum, there was marked gliosis throughout white matter tracts, most prominently in the corpus callosum. The affected dog exhibited a dramatically lower cell density in the external granular layer of the cerebral cortex compared to an age-matched normal Beagle (Fig. 1 A and B). In the affected dog, this brain layer also exhibited pronounced GFAP labeling of the astrocytes that was not observed in the brain from the normal dog (Fig.1 C and D). Substantial disease-related GFAP immunostaining was also observed in the cerebral cortical white matter tracts of the affected dog. Cerebral cortical neurons of the affected

dog also contained large aggregates of PAS-positive material in the perinuclear cell bodies (Fig. 2). The granular layer of the cerebellar folia was markedly hypocellular, and marked astrogliosis was noted throughout all layers of the cerebellar cortex and in the arbor vitae white matter as indicated by immunohistochemical staining for GFAP (Fig. 3). The retinas were not adequately prepared to evaluate for truncation of the photoreceptor outer segments as reported in a human case of PPT1 deficiency,¹⁸⁴ but dark-staining inclusions could be seen surrounding the nuclei in some retinal cells.

Based on the clinical and histological findings, neuronal ceroid lipofuscinosis (NCL) was included among the differential diagnoses. Thus, paraffin blocks, formalin-fixed tissues and EDTA-anticoagulated blood were shipped to the University of Missouri for further analysis. The NCLs are lysosomal storage diseases in which autofluorescent storage inclusions with distinct ultrastructure accumulate in the cytoplasm of neurons and other cell types. The NCLs are almost always recessively inherited and affected individuals experience progressive neurodegeneration and premature death.¹⁷¹

Fig. 1. Light micrographs of paraffin sections of the cerebral cortex external granular layer from the neuronal ceroid lipofuscinosis-affected Cane Corso (**A** and **C**) and from an age-matched normal Beagle (**B** and **D**). **A** and **B** were stained with hematoxylin and eosin. **C** and **D** were immunostained with an anti-GFAP antibody. The diseased dog exhibited a substantial reduction in cell density in the external granular layer and substantial numbers of GFAP-labeled astrocytes that were not observed in the control dog sample. Bar in (**D**) indicates magnification of all micrographs.

Fig. 2. Light micrographs of diastase-treated PAS-stained section of the cerebral cortex from the affected dog. Aggregates of PAS-staining inclusions were present in the perinuclear areas of most cortical neurons (arrows).

Fig. 3. GFAP immunohistochemical stained sections of the cerebellum from the affected Cane Corso (**A** and **C**) and from a normal Beagle of similar age (**B** and **D**). Images (**A**) and (**B**) show regions of the cerebellar cortex and images (**C**) and (**D**) show regions of the arbor vitae at the bases of the folia. In the affected dog, cells showing pronounced GFAP immunostaining were present in all layers of the cortex (m: molecular layer, p: Purkinje cell layer; g: granular cell layer) (**A**) as well as in the arbor vitae (**B**). No cells exhibited substantial GFAP immunostaining in either the cerebellar cortex or arbor vitae of the normal dog (**B** and **D**). Note also the sparsity of cell nuclei in the granular cell layer of the affected dog (**A**) compared to the normal dog (**B**).

Previously described fluorescent microscopic procedures¹⁴³ were used to examine the tissues from the affected Cane Corso. Massive accumulations of autofluorescent storage material were present throughout the brain and the retina (Fig. 4). In the cerebral cortex and midbrain almost all of the neurons contained large amounts of this material concentrated primarily in the perinuclear areas of the cells (Fig. 4A and B). In the cerebellar cortex the storage material was present

in the Purkinje cells and in large masses in the granular cell layer (Fig. 4C). Large amounts of the autofluorescent storage material were also present in the perinuclear regions of neurons in the deep cerebellar nuclei (Fig. 4D). In the retina the storage material was concentrated primarily in the ganglion cells, although small amounts of the autofluorescent material were scattered throughout the retina with a relatively large amount forming a continuous layer along the outer limiting membrane (Fig. 4E).

Small pieces of formalin-fixed cerebellar and cerebral cortex and retina were washed in sodium cacodylate buffer and then incubated in cacodylate-buffered 2.5% glutaraldehyde before being processed for electron microscopic examination. In the Purkinje cells, cerebral cortical neurons and retinal ganglion cells, the storage body contents appeared to consist primarily of aggregates of membrane-like structures. In the Purkinje cells these structures were similar in appearance to mitochondrial cristae (Fig. 5A), whereas, in the other cell types the material was much more condensed with the membrane-like profiles tightly packed together (Fig. 5B and C). Although the aggregated material formed distinct structures in all 3 tissues, membranes enclosing these aggregates were not observed except in the case of some Purkinje cell inclusions, most likely because such membranes are not well preserved with formalin fixation.

Fig. 4. Fluorescence micrographs of unstained sections of (A) cerebellar cortex, (B) cerebral cortex, (C) retina, (D) deep cerebellar nucleus, and (E) retina. In (A), arrows point to Purkinje cells; m: molecular layer, p: Purkinje cell layer, g: granular cell layer. In (C), arrows point to Purkinje cell bodies. In (E), arrows point to retinal ganglion cells and arrow heads point to the outer limiting membrane; g: ganglion cell layer, ipl: inner plexiform layer, inl: inner nuclear layer, onl: outer nuclear layer. Bar in (A) indicates magnification of all micrographs.

Fig. 5. Electron micrographs of the disease-specific storage bodies from a cerebellar Purkinje cells (A), a cerebral cortical neuron (B), and a retinal ganglion cell (C).

In conjunction with the clinical signs, the light and electron microscopic results indicated the dog had NCL. To identify the molecular genetic cause of this Cane Corso's disease, we used the dog's DNA to generate a whole genome sequence. This has proven to be an efficient strategy for identifying the mutations responsible for NCL.^{51,52,82,117,143} Procedures for DNA isolation, library preparation, sequence generation, and sequence analysis are provided in the Supplemental Material. The affected Cane Corso's whole genome sequence had 29-fold average coverage and contained 10,965 variants predicted to alter the primary structure of the encoded proteins. Only 4 of these variants occurred within one of the 13 genes (*PPT1*, *TPP1*, *CLN3*, *CLN5*, *CLN6*, *MFSD8*, *CLN8*, *CTSD*, *DNAJC5*, *CTSF*, *ATP13A2*, *GRN* and *KCTD7*)¹³⁷ associated with human NCL. Three of these variants were heterozygous missense mutations in *ATP13A2* (Table 1) and all three had previously been reported in the canine SNPdb database. The non-reference allele frequencies for these variants in 45 control whole genome sequences from normal dogs or dogs with other diseases approached or exceeded 50% and the non-reference allele was homozygous in from 15 to 19 of these control whole genome sequences (Table 1). These common sequence variants that were heterozygous in the affected dog are therefore very unlikely to have contributed to the dog's rare recessive disease.

Table 1. Sequence variants in neuronal ceroid lipofuscinosis-associated genes.

Chromosomal Coordinate ^a	Zygosity	cDNA Change	Amino Acid Change	SNPdb Reference	Frequency in Controls
g.81203298 (CFA2)	Heterozygous	<i>ATP13A2</i> :c.251A>G	p.H84R	rs85234162 7	0.485
g.81204154 (CFA2)	Heterozygous	<i>ATP13A2</i> :c.491T>C	p.M164T	rs85107807 9	0.542
g.81215472 (CFA2)	Heterozygous	<i>ATP13A2</i> :c.3469A>G	p.M1157V	rs85075182 1	0.622
g.2860424 (CFA15)	Homozygous	<i>PPT1</i> :c.124+1G>A	Splice variant	none	Zero

^a CanFam3.1 genome assembly

The remaining variant, a *PPT1*:c.124+1G>A splice donor mutation, is much more likely to be the cause of the NCL in this case. The non-reference allele was homozygous in the affected dog, has not previously been reported in dbSNP, and was absent from the whole genome sequences of 45 control canines. The Integrative Genomics Viewer genome browser (<https://www.broadinstitute.org/igv/>) was used to visualize the variant (Fig. 6) and its validity was independently verified by Sanger sequencing (details in Supplemental Materials). The *PPT1*:c.124+1G>A mutation destroys the splice donor consensus motif required for exon recognition and exon-to-exon splicing by the spliceosome.¹⁸⁵ Mutations in the "G" of the canonical "GT" or "GC" motifs found immediately 3' to all but the last exon in mammalian genes are the most common category of disease-causing splice site mutations.¹⁸⁶ Mutations that weaken or destroy splice donor sites can alter exon splicing patterns in 3 ways. They can result in the skipping of the adjacent exon, the use of nearby cryptic splice sites, or the inclusion of the intron.¹⁸⁷ The splice donor mutation in the Cane Corso with

NCL cannot induce exon skipping because it occurs adjacent to exon 1 which also contains the initiator methionine codon. A scan of the genomic DNA sequence for potential nearby cryptic sites identified 3 “GT” motifs within 50 bp of the splice donor mutation: 2 in exon 1 with starting positions at -48 and -19 relative to the intron/exon junction and 1 in intron 1 starting at position +19 relative to the intron/exon junction. However, an internet tool (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html) that estimates the strength of splice donor signals based on maximum entropy modeling of the nucleotide sequences that surround the canonical “GT” motifs¹⁸⁸ indicated that none of the 3 “GT” motifs near the splice donor mutation reside in potential cryptic splice sites (Table 2). Thus, transcripts from *PPT1* with the splice donor mutation are most likely to retain the 5’ end of the 4.6 kb intron 1. If these transcripts escaped nonsense mediated decay¹⁸⁹ and were translated, they would be expected to produce a severely truncated gene product containing 42 amino acids at the N-terminal end encoded by exon 1 followed by 114 unrelated amino acids encoded by intron 1 before the first in-frame termination codon. Nonetheless, it is highly unlikely that the mutant *PPT1* could produce a product that retained any biological function.

In 1995 *PPT1* became the first gene reported to harbor NCL-causing mutations when 3 *PPT1* mutations were identified as probable causes for NCL in 42 infants, mostly from Finland¹⁹⁰. Since then at least 70 additional variant alleles in or around *PPT1* have been reported in human NCL patients (<http://www.ucl.ac.uk/ncl/CLN1mutationtable.htm>). *PPT1* encodes a soluble

enzyme with palmitoyl-protein thioesterase activity. The human disease caused by *PPT1* mutations is referred to as CLN1 (OMIM #256730). Homozygous or compound heterozygous nullifying mutations in *PPT1* typically produce a severe form of NCL in which neurologic signs become apparent before or during the second year of life.¹⁷¹ CLN1 patients seldom survive past their early teens. Hypomorphic *PPT1* mutations have been associated with less aggressive CLN1 disease in older children and adults. Currently there are no medical treatments that can reverse or slow CLN1 disease progression.

Fig. 6. A screen capture of the affected Cane Corso's whole genome sequence alignment over 42 bp of canine chromosome 15 visualized with the Integrative Genomics Viewer. Each gray bar represents sequence reads aligned to the CanFam3.1 reference sequence assembly shown with the 4-color nucleotide sequence near the bottom of the figure. Variants in the sequence reads are indicated by letters at the pertinent positions within the gray bars. In this case, the **As** within the gray bars indicate the variant's position where an **A** in a sequence read replaces a **G** from the reference sequence. Colors were modified from the screen capture to enhance visibility of the **As**. The track at the bottom shows the amino acid sequence translated from codons at the 3' end of *PPT1* exon 1. The horizontal dashed lines enclose the homozygous **As** at position +1 of intron 1 that destroy the canonical **GT** splice donor motif.

Table 2. Palmitoyl protein thioesterase 1 exon 1 splice donor strength predictions

Position of "G" in "GT" motif	Motif	Maximum Entropy Score [†]	Conclusion
c.124+1G (wild type)	tggGTGAGC	+7.23	Potentially functional
c.124+1G>A (mutant)	tggATGAGC	-0.95	Not functional
c.77G (exonic)	ggcGTCTGG	-10.70	Not functional
c.106G (exonic)	ctgGTGATC	+0.38	Not functional
c.124+19 (intronic)	gggGTCGGA	-3.58	Not functional

[†]Maximum Entropy Scores range from -20 (weakest) to +20 (strongest). Motifs with scores above +3 are predicted to be potentially functional donor splice sites.

At least 4 different transgenic mouse models carrying nullifying *Ppt1* mutations have been created and shown to exhibit CLN1-like disease characteristics.¹⁹¹⁻¹⁹⁴

These models are the subjects of ongoing and planned evaluations of therapeutic interventions intended for human CLN1 patients.¹⁹⁵⁻²⁰⁰ Due to differences in longevity, size, and the complexities of neuroanatomy and behavior, canine NCL models have some advantages over rodent models for optimizing therapeutic interventions or establishing their efficacy. Indeed, a *TPP1*-deficient canine model for the CLN2 form of NCL has been used to develop enzyme replacement therapy¹⁸² and paved the way for ongoing human trials (<https://clinicaltrials.gov/ct2/show/NCT02678689>).

A previous report described a different homozygous canine *PPT1* mutation (*PPT1*:c.736_737insC) in a Dachshund with NCL.¹⁴⁰ The Dachshund *PPT1* frameshift mutation was predicted to encode a truncated gene product. Brain tissue from the affected Dachshund had only trace thioesterase activity, attributed to other thioesterases such as that encoded by *PPT2*.¹⁴⁰ In both the Dachshund CLN1 case and the currently described Cane Corso CLN1 case, the initial neurodegenerative changes became apparent when the dogs were approximately 8 months old. Both dogs were euthanized because of their increasingly severe neurologic signs. The Dachshund was euthanized at 14 months of age; whereas, the Cane Corso was euthanized when only 10 months old. The earlier euthanasia of the much larger Cane Corso may have been influenced by difficulties caring for a large dog with deteriorating neurologic function. The NCL in both the Cane Corso and the *PPT1*-deficient Dachshund had an early onset and rapid progression similar to that of *TPP1*-deficient Dachshunds, but was earlier and more rapid than has been reported in dogs with other types of NCL (Table 3). A similar association

between the disease-causing mutations and disease phenotype was recently confirmed by comparing the various types of human NCL.²⁰¹

Tissues from the CLN1 affected Dachshund were examined by electron microscopy.¹⁴⁰ In that case, distinct membranes were observed to at least partially surround the storage material aggregates. As with the Cane Corso tissue, the initial fixation of the Dachshund tissue was in formalin; however, the partial preservation of the surrounding membranes in the case of the Dachshund was probably due to a shorter time interval between the initial fixation and the transfer to the glutaraldehyde fixative. Better ultrastructural preservation of the storage bodies in future cases will require initial fixation in glutaraldehyde at short postmortem times. In conclusion, our findings indicate a novel splice donor mutation causing CLN1 in a dog breed previously unreported to possess the disease. A canine model for CLN1 disease could provide opportunities to further the development of therapeutic options, benefiting affected humans and canines. To find a potential founder for a colony of *PPT1*-deficient research dogs, all 5 Cane Corso DNA samples from our DNA repository and 26 Cane Corso DNA samples provided by MARS Veterinary™ were screened for the *PPT1*c.124+1A allele with a modified allelic discrimination assay that is described in the Supplemental Material. All 31 Cane Corso DNA samples tested homozygous for the reference *PPT1*c.124+1G allele. In a continuing effort to identify dogs that could help develop effective treatments for infants with CLN1 disease and to assist breeders in avoiding the production of affected puppies, we are offering free DNA testing for Cane Corsos or Dachshunds showing visual deterioration and neurodegenerative changes

starting around or before 8 months of age. Arrangements for free testing can be made with Martin Katz (katzm@health.missouri.edu).

Table 3. Ages at disease onset and euthanasia for dogs with genetically defined neuronal ceroid lipofuscinosis (NCL)

Disease	Gene	Mutation	Breed	Age at Onset (months) [†]	Age at Euthanasia (months)	Reference
CLN1	<i>PPT1</i>	<i>c.124+1G>A</i>	Cane Corso	8	10	Current
CLN1	<i>PPT1</i>	<i>c.736_737insC</i>	Dachshund	8	14	140
CLN2	<i>CLN2</i>	<i>c.325delC</i>	Dachshund	7-9	12	141
CLN5	<i>CLN5</i>	<i>c.619C>T</i>	Border Collie, Australian Cattle Dog	12-23	15-29	82,142,163
CLN5	<i>CLN5</i>	<i>c.934_935delAG</i>	Golden Retriever	13-17	30-34	143
CLN6	<i>CLN6</i>	<i>c.829T>C</i>	Australian Shepherd	18	24	144
CLN7	<i>MFSD8</i>	<i>c.843delT</i>	Chinese Crested, Chihuahua	12	16-24	51,117
CLN8	<i>CLN8</i>	<i>c.491T>C</i>	English Setter	12-14	24-27	145
CLN8	<i>CLN8</i>	<i>c.585G>A</i>	Australian Shepherd	11	21	52
CLN10	<i>CTSD</i>	<i>c.585G>A</i>	American Bulldog	11-36	42 to 66 [‡]	146,202
CLN12	<i>ATP13A2</i>	<i>c.1623G>A</i>	Tibetan Terrier	60-96	Up to 120	147,148

Supplemental material

Generation and analysis of the whole genome sequence:

The DNA was extracted from blood from the affected Cane Corso as previously described¹⁴⁵ and submitted to the University of Missouri DNA Core Facility for library preparation and sequencing. A PCR-free DNA sample preparation kit^a was used to prepare two libraries with approximate lengths of 350 bp and 550 bp. Separate 2X100 bp paired-end sequences were generated in individual flow-cell lanes with a massively parallel DNA sequencer.^b Trimmomatic²⁰³ software was used for removal of the adaptor sequences. This sequence data was deposited into the Sequence Read Archives (SRS1124447). The reads were aligned to the canine genome reference sequence (CanFam 3.1) using the default parameters of the bwa mem algorithm from the BWA 0.7.12 software.¹⁵⁷ The Samtools 1.2 software¹⁵⁸ was used to convert file formats, merge libraries, sort and index the alignment files by location. The default parameters of the Platypus 0.8.1 variant caller software¹⁵⁹ was used for variant calling. The variant annotation and filtering was done using a commercial software^c Another 45 whole genome sequences generated and processed as described above were used to estimate allele frequencies of candidate sequence variants. The breeds of origin and the SRA accession numbers for these 45 whole genome sequences are provided in the Table at the end of this section. Specific chromosomal regions of the affected Cane Corso alignment were inspected using the Integrative Genomics Viewer software to evaluate the quality of the alignment surrounding candidate sequence variants.²⁰⁴ The existence of a potentially causal homozygous variant,

PPT1:c.124+1G>A, was confirmed by direct Sanger sequencing of an amplicon produced with flanking PCR primers 5'-GCTGCCGCTGGTGATCT-3' and 5'-AGACGCGAACCCAGGTC-3'.

Genotyping individual canine DNA samples for the *PPT1:c.124+1G>A* transition:

Initial attempts to genotype individual canine DNA samples for the *PPT1:c.124+1G>A* transition with a TaqMan allelic discrimination assay⁸¹ were unsuccessful, probably due to the repetitive and GC-rich character of sequences adjacent to the candidate transition. We therefore made use of a previously described nested primer modification.²⁰⁵ A pre-amplification step was done with a commercial kit designed for PCR amplification of GC-rich templates^d The PCR primers were 5'-GGTCACGTGATCGGGCCAAGA-3' and 5'-GCCAGTCCCCGCTCCCGTCT-3' and the pre-amplification was accomplished with 18 cycles at an annealing temperature of 65o C. For the TaqMan allelic discrimination assay, 2 µl (out of the initial 30 µl pre-amplification reaction mixture) was amplified using the forward primer 5'-GCTGCCGCTGGTGATCT-3', reverse primer 5'-AGACGCGAACCCAGGTC-3'. The competing TaqMan probes were 5'-VICATGGGATGGGTGAGCGA-MGB-3' (reference allele) and 5'-FAM-ATGGGATGGATGAGCGA-MGB-3' (variant allele).

Supplemental Table 1: Breeds and SRA numbers for the control whole genome sequences

Breed	SRA Number
Standard Poodle	SRS932171
Airedale	SRS932170
Basenji	SRS1124466
Soft Coated Wheaten Terrier	SRS834052
Kerry Blue Terrier x beagle cross	SRS1124465
Labrador Retriever	SRS1124463
Chinook	SRS932169
Pembroke Welsh Corgi	SRS732549
Pembroke Welsh Corgi	SRS732550
Pointer	SRS932168
Jack Russell Terrier	SRS932167
Italian Greyhound	SRS932166
Saint Bernard	SRS932165
Portuguese Podengo	SRS932163
Border Collie	SRS932162
English Springer Spaniel	SRS932160
German Shepherd	SRS932159
Basenji	SRS932158
Labrador Retriever	SRS932152
Jack Russell Terrier	SRS932151
Tibetan Terrier	SRS932148
Jack Russell Terrier	SRS932147
Berger Picard	SRS932146
Tibetan Terrier	SRS932145
Border Terrier	SRS932144
Standard Poodle	SRS932143
Shetland Sheepdog	SRS932138
Berger Picard	SRS1124462
Airedale Terrier	SRS1124461
Soft Coated Wheaten Terrier	SRS834054
Jack Russell Terrier	SRS1124459
English Cocker Spaniel	SRS834057
Black & Tan Coonhound	SRS1124458
Golden Retriever	SRS1124457
Border Terrier	SRS1124456
Border Terrier	SRS1124455
English Springer Spaniel	SRS1124454
English Setter	SRS1124453
Gordon Setter	SRS1124452
Berger Picard	SRS1124451
Shiba Inu	SRS1124450
Labrador Retriever	SRS1124449
Golden Retriever	SRS834021
Australian Cattle Dog	SRS834022
Lowchen	SRS1124448

Footnotes

a TruSeq DNA PCR-Free Sample Preparation Kit, Illumina, San Diego, CA

b HiSeq 2500 System, Illumina, San Diego, CA

c SNP & Variation Suite v8.x, Golden Helix, Inc., Bozeman, MT

d AccuPrime™ GC-rich kit, Invitrogen, Carlsbad, CA 92008, USA

Chapter 5

GM2 gangliosidosis in Shiba Inu dogs with an inframe deletion in *HEXB*

Abstract

Consistent with a tentative diagnosis of neuronal ceroid lipofuscinosis (NCL), autofluorescent cytoplasmic storage bodies were found in neurons from the brains of 2 related Shiba Inu dogs with a young-adult onset, progressive neurodegenerative disease. Unexpectedly, no potentially causal NCL-related variants were identified in a whole genome sequence generated with DNA from 1 of the affected dogs. Instead, the whole genome sequence contained a homozygous 3 base pair (bp) deletion in a coding region of *HEXB*. The other affected dog also was homozygous for this 3 bp deletion. Mutations in the human *HEXB* ortholog cause Sandhoff disease, a type of GM2 gangliosidosis. Thin layer chromatography confirmed that GM2 ganglioside had accumulated in an affected Shiba Inu brain. Enzymatic analysis confirmed that the GM2 gangliosidosis resulted from a deficiency in the *HEXB* encoded protein and not from a deficiency in products from *HEXA* or *GM2A*, which are known alternative causes of GM2 gangliosidosis. We conclude that the homozygous 3 bp deletion in *HEXB* is the likely cause of the Shiba Inu neurodegenerative disease and that whole genome sequencing can lead to the early identification of potentially disease-causing DNA variants thereby refocusing subsequent diagnostic analyses towards confirming or refuting candidate variant causality.

Introduction

The clinical records of 2 young-adult Shiba Inu dogs that exhibited similar signs of progressive, fatal, neurodegeneration came to our attention from independent sources. Pedigree inspection indicated that both dogs shared at least 1 common maternal and paternal ancestor, as illustrated in Fig. 1. Thus, it appeared that the same recessively inherited neurodegenerative disease affected both dogs. We used whole genome sequencing to efficiently identify a genetic variant that was most likely responsible for this disease and to reach a highly probable, but unexpected, diagnosis.

Fig.1 Pedigree shows consanguineous familial relationships for the 2 affected Shiba Inu, Dog A and Dog B. The dog marked with an asterisk contributed genetic material to both parents of both affected dogs. Bold lines show the shortest possible pathway for the inheritance of the causal allele from an ancestor to the homozygous affected dogs. Nonetheless, we do not have DNA from the ancestors to verify this pathway and additional consanguineous relationships among earlier ancestors are likely. Thus, Dog A and Dog B may have obtained their homozygous mutant allele by alternative inheritance pathways.

Methods

The DNA from 1 affected Shiba Inu (Dog A) was submitted to a commercial sequencing center^a for PCR-free library preparation and sequencing with a commercial sequencer^b that produced paired-end 150 bp reads. Details about the pipeline used to process and analyze this data set are available in the supplementary material. The PCR primers 5'-AAATAGTATAATCATGTGCAA-3' and 5'-CCCAAATACATTTACTGCCTA-3' were used to produce amplicons containing a candidate variant identified in the whole genome sequence. These amplicons were analyzed by Sanger sequencing for validation and by PCR-restriction fragment length polymorphism (PCR-RFLP) with MnlI for genotyping archived Shiba Inu DNA. Previously described procedures were used for fluorescence and electron microscopy⁵² and for thin layer chromatography and enzymology.¹¹⁷

Clinical Findings

Dog A, a spayed female Shiba Inu, exhibited progressive neurologic signs starting with generalized tremors and altered tail carriage (tail down) when approximately 12 months old. As the disease progressed, the severity of the tremors increased and the dog exhibited marked cerebellar ataxia, spastic tetraparesis, anxiety, decreased appetite, decreased responsiveness to verbal commands, trance-like behavior, loss of ability to climb up and down stairs, and impaired vision. Magnetic resonance imaging (MRI) of the head showed a diffuse loss of gray-white matter distinction on T2-weighted imaging with white matter appearing isointense to

marginally hypointense relative to grey matter. Because of the progression of neurological signs, the dog was euthanized at approximately 17 months of age.

Dog B also was a spayed female Shiba Inu. Similar to Dog A, the first clinical signs in Dog B appeared at about 12 months of age and included situational anxiety, inability to climb up or down stairs, a wide-based stance, severe generalized tremors, and the same alteration in tail carriage shown by Dog A. During a neurologic examination at 15 months of age, Dog B had normal proprioception but showed intention tremors and severe cerebellar ataxia with frequent falls. The menace response was decreased to absent. A brain MRI, including T2- and T1-weighted sequences before and after administration of a contrast agent, showed decreased gray-white matter distinction in both the cerebrum and cerebellum similar to Dog A. In the cerebellum, arbor vitae could not be identified on the sagittal T2-weighted sequence. The ventricular system was symmetrical and normal in size. The neurological signs progressed to include anxiety, compulsive panting, trance-like behavior, intolerance to loud noise, and loss of training including house training. Dog B developed severe muscle stiffness and weakness that spread from the hind limbs resulting in ataxia and frequent falls. In addition, Dog B showed severe visual impairment in both bright and dim light. A sleep disturbance with leg movements mimicking running started at about 18 months of age. Near the end of its life, Dog B was unable to walk or eat without assistance. Dog B died while asleep when approximately 20 months old. Commercial laboratories^{c,d} determined that neither affected dog had a *GLB1* frameshift

mutation, chr23:3,796,317delC (CanFam 3.1), known to cause GM1 gangliosidosis in the Shiba Inu breed (OMIA [000402-9615](#)).²⁰⁶

Because the clinical signs suggested neuronal ceroid lipofuscinosis (NCL) as a possible cause, blood and other tissue samples were shipped to the University of Missouri for further analysis. Fluorescence microscopy of unstained tissue sections from both affected dogs identified massive accumulations of yellow-emitting autofluorescent storage bodies in neurons throughout the cerebellum (Fig. 2A), the cerebral cortex (Fig. 2B) and the retina (Fig. 2C).

Fig. 2 Fluorescence micrographs of unstained sections of cerebellum (**A**), cerebral cortex (**B**), and retina (**C**) from an affected Shiba Inu. In the cerebellum, autofluorescent intracellular inclusions were present in both the Purkinje cell (p) and molecular (m) layers. Neurons throughout the cerebral cortex gray matter had abundant autofluorescent inclusions. In the retina, the most pronounced accumulation of autofluorescent material was seen in the ganglion cell layer (g), but some accumulation was also present in the inner nuclear layer (inl). Bars in (**A**) and (**B**) indicate the magnification for the micrographs.

Molecular-genetic diagnosis

The clinical signs and fluorescent microscopic results were consistent with a diagnosis of NCL. Because whole genome sequencing previously had proven to be an effective strategy for identifying genetic variants responsible for NCL in dogs,^{51,52,54,82,117,143} a whole genome sequence was generated with DNA from Dog A. The resulting sequence data had 31-fold average coverage of the CanFam3.1 reference assembly and is available in the National Center for Biotechnology Information Sequence Read Archives (accession SRS1692083). As we have done previously,^{52,82,117,143} the aligned reads from the whole genome sequence were scanned for rare variants in or near the coding exons of canine orthologs of the 13 genes currently associated with human NCL: *PPT1*, *TPP1*, *CLN3*, *CLN5*, *CLN6*, *MFSD8*, *CLN8*, *CTSD*, *DNAJC5*, *CTSF*, *ATP13A2*, *GRN* and *KCTD7*.¹³⁷ Unexpectedly, we did not find plausible candidate variants. In addition, the alignment was checked for a *GLB1* frameshift mutation, chr23:3,796,317delC (CanFam 3.1), known to cause a GM1 gangliosidosis in the Shiba Inu,²⁰⁶ but only the reference allele was found confirming antemortem DNA test results. The search for a causal genetic variant was expanded by filtering the millions of sequence variants found in the Shiba Inu whole genome sequence to retain only those that were: (1) predicted to affect the primary structure of a gene product, (2) homozygous in the affected Shiba Inu, and (3) absent from the 105 whole genome sequences of normal dogs or dogs with other diseases. Only 80 variants in 74 different genes met these criteria (see Supplementary Table 1). Brief searches for the known biological functions of these 74 genes in OMIM

(<https://www.ncbi.nlm.nih.gov/omim>) and in PubMed (<https://www.ncbi.nlm.nih.gov/pubmed>) identified a 3 bp deletion in *HEXB* as the sequence variant considered most likely to be responsible for the Shiba Inu disorder because it was the only variant in a gene that has previously been associated with lysosomal storage disease. The chromosomal location of this deletion is chr2:57,243,656_57,243,658delCCT (CanFam 3.1).

Sanger sequencing confirmed that both affected Shiba Inu were homozygous for the 3 bp *HEXB* deletion. The absence of this deletion from our 105 control whole genome sequences suggested that it is uncommon in dogs in general. To estimate the frequency of the 3 bp *HEXB* deletion among the Shiba Inu, all 40 Shiba Inu represented in the University of Missouri DNA repository were genotyped by PCR-RFLP for this deletion. Thirty-seven of the tested Shiba Inu were homozygous for the reference allele; the other 3 were heterozygotes. These heterozygotes were born in 2002, 2004, and 2007. There are no known familial relationships among them or between them and the 2 affected Shiba Inu described here. This observation suggests that the 3 bp *HEXB* deletion may be rare but widely distributed in the Shiba Inu breed.

Biochemical and electron microscopic confirmation

The *HEXB* deletion predicts the loss of a leucine residue (p.Leu317del; XP_005617969.1) in the encoded β -subunit of the β -hexosaminidase enzyme complex. A leucine residue occurs at a homologous position in β -subunits from 53 other mammalian species (see Supplementary Figure 1). A functional β -subunit

provides essential glycosidic activity to the β -hexosaminidase enzyme complex that removes the terminal N-acetyl-galactosamine from GM2 ganglioside, an essential step in the lysosomal degradation of gangliosides. Genetic variants in human *HEXB* can result in a β -hexosaminidase deficiency called Sandhoff disease and cause the accumulation of GM2 ganglioside.²⁰⁷

Thin layer chromatography was used to determine if GM2 ganglioside had accumulated in brain gray matter from 1 of the affected Shiba Inu (Dog B). The resulting thin layer chromatogram is shown in Fig. 3. An extract from the brain of a human patient with GM2 gangliosidosis known to contain GM2 ganglioside as the major component was fractionated in Lane 1. The brain extract in lane 3 was from a human GM1 gangliosidosis patient. GM1 ganglioside was the major component in this extract. Lane 4 contained a normal bovine brain extract and served to show that GM1 and GM2 gangliosides do not accumulate in healthy brains. The extract in lane 2 was from one of the affected Shiba Inu (Dog B). The migration of the major component in this lane matched that in lane 1, indicating that it is also GM2 ganglioside and that the dog had a GM2 gangliosidosis.

Fig.3 Thin layer chromatographic fractionation of brain gangliosides extracted from the gray matter of a human patient who died from Tay-Sachs disease (lane 1), the gray matter of Dog B, the affected Shiba Inu dog (lane 2), the gray matter of a human patient who died with GM1 gangliosidosis (lane 3) and gangliosides extracted from a normal bovine brain obtained from a commercial source^e (lane 4). The most abundant component in lane 1 is GM2 ganglioside (GM2). In lane 2, the most abundant component co-migrated with the GM2 ganglioside in lane 1 and not with GM1 ganglioside (GM1), the most abundant component in lane 3. Only trace amounts of GM2 ganglioside were detected in Lane 3 and none was detected in extracts from the normal brain in lane 4.

Besides *HEXB*, GM2 gangliosidosis can be caused by genetic variants in 2 other genes: *HEXA* that encodes the α -subunit of the β -hexosaminidase enzyme complex and *GM2A* that encodes GM2 activator protein. Although the activator protein does not possess intrinsic enzymatic activity, its presence is required for efficient enzymatic degradation of GM2 ganglioside by β -hexosaminidase.²⁰⁸ The α - and β -subunits of β -hexosaminidase are enzymatically active as homo- or heterodimers. The 3 genetic causes of GM2 gangliosidosis can be enzymologically

distinguished with synthetic substrates that identify the source of the residual hexosaminidase activities in patients' tissues.²⁰⁷ Patients with GM2 gangliosidosis caused by a *GM2A* deficiency have residual activity from both the α - and β -subunits. Thus, they are classified as AB variants. Human patients with *HEXA* deficiency (known as Tay-Sachs disease) are considered B variants because they have only β -subunit activity. Homodimers of the α -subunit are unstable and heat labile. Patients with Sandhoff disease (*HEXB* deficiency) who lack the β -subunit necessary to form stable heterodimers, can only form unstable α -subunit homodimers and thus have subnormal activities from both the α - and β -subunits.²⁰⁷ These patients are considered to be 0 (zero) variants. Table 1 contains a summary of the genetic causes, the subunit activities and the associated nomenclature for the 3 causes of GM2 gangliosidosis.

Table 1: Types of GM2 Gangliosidosis

Disease	Gene [†]	Encoded Protein	Residual Activity	Variant [‡]
Tay-Sachs Disease	<i>HEXA</i>	α -Subunit of β -Hexosaminidase	β -Subunit	B Variant
Sandhoff disease	<i>HEXB</i>	β -Subunit of β -Hexosaminidase	None [*]	0 Variant
GM2 Activator Deficiency	<i>GM2A</i>	GM2 Activator	α - and β -Subunits	AB variant

[†]Harboring causal variants; [‡]Based on residual activity; ^{*}None because α -Subunit dimers are unstable

We used previously described procedures²⁰⁹ and the synthetic substrate, 4-methylumbelliferyl N-acetyl- β -d-glucosaminide, to estimate total β -hexosaminidase activity in cerebral cortical gray and white matter extracts. We also measured β -galactosidase activity in these extracts as a control. As indicated in Table 2, the total β -hexosaminidase activities in the extracts from Dog B were

markedly decreased compared to 2 control dogs. Furthermore, the β hexosaminidase activities in the extracts from Dog B were completely heat labile as would be expected for α -subunit homodimers. These results are similar to those observed with 0 variant or Sandhoff disease patients and provide further evidence that the *HEXB* deletion identified by whole genome sequencing is responsible for the progressive neurodegeneration in the 2 affected Shiba Inu dogs.

Table 2: Enzyme Activities

Enzyme	Enzymatic Activities (nmol/h/mg protein)					
	Affected Dog B		Control Dog 1		Control Dog 2	
	Gray Matter	White Matter	Gray Matter	White Matter	Gray Matter	White Matter
β -galactosidase [†]	269	174	67.1	25.5	58.9	31.8
β -hexosaminidase (% Heat labile)	15.1(10 0%)	11.3 (100%)	663 (77%)	363 (72%)	669 (79%)	427(79%)

[†]Run as control

Electron microscopic examination of the storage bodies in the brain and retina showed that many neurons contained membrane-like structures arranged in loosely associated concentric whorls (Fig. 4A, C and D). Inclusions with this ultrastructural feature traditionally are called membranous cytoplasmic granules.^{207,208,210,211} Other less common neuronal inclusions appeared to be vacuoles with only a small amount of dispersed electron-dense material (Fig. 4B). Abnormal inclusions with the membranous cytoplasmic granules ultrastructure are common in human patients with GM2 gangliosidosis.^{210,212,213} In addition, membranous storage granules have been found in neurons from patients with GM1 gangliosidosis or mucopolysaccharidosis.^{213,214} However, they have not been reported in NCL-affected brains, which instead typically contain inclusions with

granular, curvilinear, rectilinear, or fingerprint profiles, either singularly, or in combination.²¹⁵

Fig.4 Electron micrographs of storage material in the cerebral cortex (**A** and **B**), cerebellar molecular layer cell (**C**), and retinal ganglion cells (**D**) of Dog B. Representative storage bodies are indicated by arrows. In cerebral cortical neurons, the storage material consisted of both concentric whorls of membrane-like structures (**A**) and membrane-bound vacuole-like inclusion bodies (**B**). In the cerebellum (**C**), the storage bodies contained more irregularly arranged membrane-like material. The ultrastructural appearance of the cerebellar storage bodies was similar in both the molecular layer and Purkinje cells. In the retinal ganglion cells (**D**), the storage material consisted primarily of concentric whorls of membrane-like structures.

Autofluorescent storage bodies in NCL and other diseases

Before we evaluated the whole genome sequence, we suspected that the affected Shiba Inu dogs had NCL because of their clinical signs of progressive neurodegeneration and the presence of autofluorescent storage bodies. Progressive neurodegeneration and accumulation of autofluorescent lysosomal storage bodies within neurons are widely recognized disease characteristics required for inclusion among the diverse diseases classified as NCLs.¹³⁷ Nonetheless, in a few cases, disorders with these characteristics have been classified as other types of lysosomal storage diseases because of the biochemical composition of the associated storage granules or the nature of the associated enzyme deficiency or both. Among these are GM1 gangliosidosis and mucopolysaccharidosis IIIA in humans and α -mannosidosis in mice.^{211,214,216,217} In addition, there is an example in dogs. When American Staffordshire Terriers and Pit Bull Terriers with a recessively inherited, adult-onset, progressive neurodegenerative disease were found to accumulate autofluorescent storage bodies in hippocampal and cerebellar neurons, their disease was classified as an NCL.²¹⁸ This classification remained unchanged with the identification of the causal genetic variant in *ARSG*, a gene with a biological function that had not yet been determined.²¹⁹ Subsequently, it was shown using *Arsg*-knockout mice that the natural substrate for the encoded enzyme was heparan sulfate, a mucopolysaccharide, and that the storage bodies were rich in partially degraded heparan sulfate.²²⁰ Thus, the disease caused by *ARSG* deficiency is now considered to be a mucopolysaccharidosis. Similarly, the Shiba Inu of our case

report were thought to have NCL until we determined that brain extracts from 1 of the dogs contained accumulated GM2 ganglioside and had a β -hexosaminidase deficiency, thus classifying the disorder as a gangliosidosis.

Ours may be the first report that storage bodies associated with GM2 gangliosidosis are autofluorescent. In fact, at least 1 report states that storage bodies from human patients with GM2 gangliosidosis do not autofluoresce.²¹⁰ Thus, we examined tissue from a previously reported case of GM2 gangliosidosis in a Japanese Chin dog caused by a *HEXA* mutation (OMIA [001461](#)-9615).²⁰⁹ As with the results reported here, the storage bodies in the tissue from the dog with the *HEXA* mutation were autofluorescent (Fig. 5).

Fig. 5 Fluorescence micrographs of unstained cryostat sections of cerebellum (**A**) and cerebral cortex (**B**) from a Japanese Chin dog with GM2 gangliosidosis due to a homozygous *HEXA* nonsynonymous substitution. In the cerebellum, autofluorescent inclusions were present primarily in the Purkinje cells (arrows in A) in the Purkinje cell layer (p), although weakly fluorescent cells were also present in the molecular layer (m). No autofluorescence was observed in the granular cell layer (g). Neurons throughout the cerebral cortex gray matter of the parietal lobe had abundant autofluorescent inclusions (arrows in B). Bar in (**A**) indicates the magnification for micrographs (**A**) and (**B**).

Comparison with other gangliosidoses in dogs

Although a GM1 gangliosidosis has been recognized in the Shiba Inu for >15 years,^{206,221,222} ours is the first report of a GM2 gangliosidosis in this breed. A GM2 gangliosidosis has been reported in a mixed breed dog and in members of other dog breeds including German Shorthaired Pointer, Japanese Chin, Golden Retriever, and Toy Poodle.^{209,223-227} The Toy Poodle, Golden Retriever and the mixed breed dog were classified as 0 variants (OMIA [001462-9615](#)).²²⁵⁻²²⁷ The 1 bp *HEXB* frameshift variant responsible for the 0-variant gangliosidosis in Toy Poodles²²⁸ is different from the 3 bp in-frame deletion that we found in the affected Shiba Inu dogs. The molecular genetic causes for the 2 other 0-variant gangliosidoses have not been reported.

Conclusions

Although not yet routine, the sequencing and analysis of whole genomes generated from patient DNA is becoming an increasingly important strategy for diagnosing inherited diseases in humans.²²⁹ Technical improvements in massively parallel DNA sequencing have resulted in the development of a sequencing instrument^b capable of generating deep sequence coverages of mammalian genomes for <\$2,000.²³⁰ Although initially licensed exclusively for the sequencing of human genomes,^{231,232} this instrument can now be used to sequence domestic animal genomes. With access to this technology, it became economically feasible for us to make use of whole genome sequencing early in our efforts to identify the

molecular genetic cause of the neurodegeneration in these Shiba Inu. Analysis of the whole genome sequence from 1 of the affected dogs identified a potentially causal candidate variant. This finding enabled us to proceed with more traditional diagnostic techniques (e.g., thin layer chromatography, enzymology, electron microscopy) that were chosen specifically to confirm or refute the causality of the candidate variant. Thus, in our case study, whole genome sequencing was an efficient and cost effective procedure that led to the identification of the Shiba Inu disease as a GM2 gangliosidosis caused by a 3 bp deletion in *HEXB*. Once impediments such as long turn-around times, low causal variant discovery rates and inadequate data storage facilities have been overcome, we expect whole genome sequencing to become an increasingly important tool for diagnosing heritable animal diseases.

Footnotes

^aMcDonnell Genome Institute, Washington University, St Louis, MO 63108, USA

^bHiSeq X Sequencing System, Illumina, Inc., San Diego, CA 92122, USA^cPaw Print Genetics, 850 E. Spokane Falls Blvd. Suite 200, Spokane, WA 99202, USA

^d**VetGen**, 3728 Plaza Drive, Suite 1, Ann Arbor, MI 48108, USA

^eSigma-Aldrich, 3300 S 2nd St, St. Louis, MO 63118, USA

Supplemental Material

Whole Genome Sequence Data Processing Pipeline

DNA from an affected Shiba Inu was submitted to a commercial sequencing center for PCR-free library preparation and massively parallel DNA sequencing with a commercial sequencer^b using paired end 150 bp reads. The sequence data went through an initial quality control, which included removing adapters using a custom Perl script and examination of overall sequence quality using FastQC (v0.11.5). Reads were trimmed based on quality values using trimmomatic (version 0.35) and then aligned to the canine reference sequence assembly CanFam3.1 using BWA (v0.7.1). The alignment files were sorted and indexed using Samtools (v1.3.1). Picard (v2.6.0) was used to merge alignment files and mark duplicate sequences by library. Base quality score recalibration, indel realignment and variant calling using HaplotypeCaller were performed using GATK (v3.6) according to best practices. Variant annotation and filtering were performed with commercial software.^c Annotation was accomplished with RefSeqGenes (NCBI release 104 – 2016-05-23). Similarly generated whole genome sequences from dogs with other diseases (n = 102) or from healthy dogs (n = 3) served as controls.

a McDonnell Genome Institute, Washington University, St Louis, MO 63108, USA

b HiSeq X Sequencing System, Illumina, Inc., San Diego, CA 92122, USA

Supplementary Figure 1. Alignments of β -hexosaminidase β -subunit amino acids sequences from 54 mammalian species predicted from codons surrounding the deleted Leu137 codon in the affected Shiba Inu

Consensus	A	R	X	R	G	I	R	V	X	P	E	F	D	X	P	G	H	T	X	S	W	G	K	G	O	X	L	L	T	P	C	Y	X	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X			
Identity	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
1. Normal dog	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
2. Affected dog	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
3. African savanna elephant	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
4. Alpaca	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
5. American bobcat	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
6. American mink	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
7. American pika	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
8. Amur tiger	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
9. Arabian camel	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
10. Donkey	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
11. Bactrian camel	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
12. Big brown bat	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
13. Black flying fox	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
14. Black shub-nosed monkey	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
15. Bolivian squirrel monkey	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
16. Indian cattle	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
17. Bottlenose dolphin	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
18. Cape elephant shrew	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
19. Cape golden mole	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
20. European cattle	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
21. Chetan	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
22. Chimpanzee	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
23. Chinese hamster	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
24. Chinese rufous horseshoe bat	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
25. Chinese tree shrew	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
26. Crab-eating macaque	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
27. Domestic cat	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
28. Domestic ferret	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
29. Domestic guinea pig	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
30. Drill	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
31. Giant panda	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
32. Goat	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
33. Golden-collared manakin	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
34. Golden shrew	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
35. Golden hamster	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
36. Gray mouse lemur	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T	Q	S	W	G	K	G	K	N	N	L	L	T	T	P	C	Y	X	X	X	X	X	X	G	P	X	N	P	X	X	N	X	T	Y	X	F	X	X	X	F	X
37. Gray short-tailed opossum	A	R	L	R	R	G	I	R	V	I	P	E	D	S	P	P	G	H	T	T																																								

Supplemental Table 1: Rare variants predicted to change the primary sequence of the gene product for which the affected Shiba Inu was predicted to be homozygous

Coordinate	Gene	Nucleotide change	Consequence
1:924522	<i>CTDP1</i>	c.1990C>A	Nonsyn SNV
1:104890731	<i>LOC106558999</i>	c.-56+1_-56+2insT	Splicing
2:33828898	<i>ADARB2</i>	c.1879G>A	Nonsyn SNV
2:42310546	<i>SPINK5</i>	c.293A>T	Nonsyn SNV
2:43248005	<i>IL6ST</i>	c.12G>T	Nonsyn SNV
2:48844920	<i>IPO11</i>	c.2096G>A	Nonsyn SNV
2:48844931	<i>IPO11</i>	c.2107C>A	Nonsyn SNV
2:52647551	<i>CD180</i>	c.646C>T	Nonsyn SNV
2:57243653	HEXB	c.945_947delCCT	Del
2:58350523	<i>CCDC113</i>	c.913G>A	Nonsyn SNV
2:58579428	<i>CNGB1</i>	c.214C>T	Nonsyn SNV
2:58781765	<i>ADGRG3</i>	c.1595C>T	Nonsyn SNV
2:65342376	<i>LOC487290</i>	c.203G>A	Nonsyn SNV
2:76408401	<i>LACTBL1</i>	c.677C>T	Nonsyn SNV
3:18314343	<i>ADGRV1</i>	c.3502C>T	Nonsyn SNV
4:57953189	<i>SLC36A2</i>	c.958C>T	Nonsyn SNV
4:59883859	<i>SH3TC2</i>	c.1273G>A	Nonsyn SNV
6:8811313	<i>MUC17</i>	c.1294_1295insAGACGTGACCACATCATCAGTA TCTCCAGATGGGACCACATCAGCAGCTGCTC CA	Stopgain
6:12571677	<i>TNRC18</i>	c.4857C>G	Nonsyn SNV
6:13248516	<i>SDK1</i>	c.2912G>T	Nonsyn SNV
6:15276899	<i>ELFN1</i>	c.1886C>T	Nonsyn SNV
6:15413161	<i>TMEM184A</i>	c.166C>T	Nonsyn SNV
6:36540441	<i>PPL</i>	c.4405C>T	Nonsyn SNV
6:36661974	<i>ZNF500</i>	c.1361G>A	Nonsyn SNV
6:39118587	<i>IGFALS</i>	c.1900G>A	Nonsyn SNV
6:39121141	<i>NUBP2</i>	c.37G>A	Nonsyn SNV
6:39478018	<i>LOC448801</i>	c.116C>T	Nonsyn SNV

Coordinate	Gene	Nucleotide change	Consequence
6:39511373	<i>CACNA1H</i>	c.6328G>T	Nonsyn SNV
6:39518309	<i>CACNA1H</i>	c.4445G>A	Nonsyn SNV
6:39978234	<i>PIGQ</i>	c.292G>T	Nonsyn SNV
6:39984223	<i>NHLRC4</i>	c.1259G>A	Nonsyn SNV
6:39984362	<i>NHLRC4</i>	c.1120G>A	Nonsyn SNV
6:39984617	<i>NHLRC4</i>	c.865G>A	Nonsyn SNV
6:40429609	<i>LOC611642</i>	c.278G>A	Nonsyn SNV
6:40654696	<i>LOC611675</i>	c.993_994insCTGCCTCCATCTACCTATGTTCTT AATGTATGTATACACCCCAGG	Ins
6:40823680	<i>DENND2D</i>	c.264G>T	Nonsyn SNV
9:36330268	<i>CA4</i>	c.412A>G	Nonsyn SNV
11:3522330	<i>LOC474642</i>	c.328C>T	Stopgain
11:12337210	<i>SNCAIP</i>	c.997C>G	Nonsyn SNV
14:40359259	<i>HOXA11</i>	c.664_665insCGG	Ins
14:43195886	<i>NOD1</i>	c.1534G>A	Nonsyn SNV
15:1570206	<i>HIVEP3</i>	c.3269C>T	Nonsyn SNV
15:3345340	<i>LOC100855618</i>	c.1268C>T	Nonsyn SNV
15:5952737	<i>SH3D21</i>	c.308G>A	Nonsyn SNV
15:6922899	<i>ZMYM1</i>	c.319G>T	Nonsyn SNV
15:7005024	<i>ZMYM6</i>	c.2847A>G	Nonsyn SNV
15:8995338	<i>ORC1</i>	c.2554G>A	Nonsyn SNV
15:15536284	<i>BEST4</i>	c.479C>T	Nonsyn SNV
15:16132552	<i>KLF18</i>	c.1367A>T	Nonsyn SNV
15:17495861	<i>LOC100855686</i>	c.40C>T	Nonsyn SNV
15:17763635	<i>KLHL33</i>	c.565G>C	Nonsyn SNV
15:18124446	<i>TPPP2</i>	c.479G>A	Nonsyn SNV
15:22772701	<i>LOC106559776</i>	c.39_41delTGT	Del
15:51916068	<i>DCHS2</i>	c.9953C>T	Nonsyn SNV
16:5993505	<i>COR13M1</i>	c.125A>C	Nonsyn SNV
16:20282728	<i>PTPRN2</i>	c.1516G>A	Nonsyn SNV

Coordinate	Gene	Nucleotide change	Consequence
18:26154975	<i>LOC106559966</i>	c.443G>C	Nonsyn SNV
18:46124790	<i>LOC102153419</i>	c.938G>A	Nonsyn SNV
18:53442696	<i>LGALS12</i>	c.407T>C	Nonsyn SNV
22:52246650	<i>CCDC168</i>	c.2524C>T	Nonsyn SNV
22:52356145	<i>ERCC5</i>	c.1658C>T	Nonsyn SNV
23:8212869	<i>ACVR2B</i>	c.431C>T	Nonsyn SNV
23:8790242	<i>TTC21A</i>	c.782A>C	Nonsyn SNV
23:8853006	<i>XIRP1</i>	c.2857C>T	Nonsyn SNV
24:44550537	<i>PPP1R3D</i>	c.683G>A	Nonsyn SNV
25:35432289	<i>LOC106557768</i>	c.556delT	Frameshift Del
25:35432291	<i>LOC106557768</i>	c.558_559delCC	Stopgain
25:37253297	<i>ZNF596</i>	c.157A>G	Nonsyn SNV
27:1770147	<i>PRR13</i>	c.344A>T	Nonsyn SNV
27:2448245	<i>KRT2</i>	c.392G>C	Nonsyn SNV
27:38938186	<i>VWF</i>	c.6380C>T	Nonsyn SNV
31:29813863	<i>C31H21orf140</i>	c.738_740delCTA	Del
31:38348044	<i>LOC100685181</i>	c.241G>A	Nonsyn SNV
31:38355090	<i>LOC611489</i>	c.568_569insCACCTCCA	Frameshift Ins
33:29155279	<i>TNK2</i>	c.1597G>A	Nonsyn SNV
36:3337579	<i>GALNT5</i>	c.1166A>G	Nonsyn SNV
X:1454947	<i>XG</i>	c.358G>A	Nonsyn SNV
X:1459432	<i>XG</i>	c.399C>G	Nonsyn SNV
X:6822143	<i>WWC3</i>	c.2382G>C	Nonsyn SNV
X:77011557	<i>TCEAL4</i>	c.250_270delAAGCCAGCGGAGAGCGAGGGG	Del

Chapter 6

Discussion and Conclusion

The previous chapters (2 to 5) presented four examples in which my thesis-related research efforts lead to reportable discoveries; however, the research described in those chapters represents only a small fraction of my entire research effort since joining the Dr. GS Johnson molecular genetics laboratory as a graduate student in 2013. This final chapter presents a more balanced picture of my overall research efforts, starting with a brief description of the research environment and available resources in the Johnson laboratory, from when my research began and up until now. Next, I present my personal experiences with different procedures for generating and interpreting the canine whole genome sequences and how they advanced during my years as a graduate student. After that, examples of strategies that were not always successful in identifying disease-causing mutations are given, as are examples of efforts to match apparently deleterious sequence variants with suspected phenotypes unrelated to the dog's primary disease. Last, are a brief description of a successful causal gene identification discovered after the writing of this thesis had already started, and suggestions to successfully conclude other ongoing canine disease investigations.

Dr. GS Johnson's molecular genetic laboratory at the University of Missouri focuses on detecting variants associated with disease phenotypes in dogs. Currently, the lab has a canine DNA repository with over 180,000 samples from multiple breeds that were accumulated over three decades of research. From 1990

until 2010, 17 disease associated variants were identified using traditional methods, such as mapping and candidate gene exon sequencing. In 2010, NGS started to become affordable for canine disease research and since then the lab has submitted DNA samples for WGS for 96 dogs from 53 different breeds and about 40 different disease phenotypes. Together with collaborating institutes, over 500 canine WGS are available for data analysis. The causal mutations for 20 disease phenotypes have been successfully identified through WGS analysis between 2010 and now in our lab, including the four mutations discussed in chapters 2, 3, 4 and 5 (Table 1). To date, all of these successes involve recessive Mendelian traits, and most of the associated mutations are coding (exonic), with the exception of the Cane Corso *PPT1* mutation, which is within a splice donor site. Most of the samples that were submitted for WGS are thought to be rare recessive Mendelian traits based on pedigree or previous knowledge (e.g. lysosomal storage diseases), with some possible exceptions, such as epilepsy, which is now thought to be a polygenic trait in most cases.

Table 4. Successfully identified causal mutations using WGS data.

Breed	Disease	Gene
Chinese crested	Multiple system degeneration	<i>SERAC1</i> *
Basenji	Fanconi syndrome	<i>FAN1</i> *
Soft coated wheaten terrier	Paroxysmal dyskinesia	<i>PIGN</i> ⁸⁰
Jack russell terrier	Spinocerebellar ataxia	<i>KCNJ10</i> ¹¹⁶
Kerry blue terrier	Multiple system degeneration	<i>SERAC1</i> *
Basenji	Progressive retinal atrophy	<i>SAG</i> *
Chinese crested	Neuronal ceroid lipofuscinosis	<i>MFSD8</i> ¹¹⁷
Standard schnauzer	Dilated cardiomyopathy	<i>RBM20</i> *
Australian shepherd	Neuronal ceroid lipofuscinosis	<i>CLN8</i> ⁵²
Black russian terrier/Rottweiler	Laryngeal paralysis/spongiform encephalopathy	<i>RAB3GAP1</i> ^{23,3234}
English cocker spaniel	Peripheral neuropathy	<i>STK11IP</i> *
Golden retriever	Neuronal ceroid lipofuscinosis	<i>CLN5</i> ¹⁴³
Australian cattle dog	Neuronal ceroid lipofuscinosis	<i>CLN5</i> ⁸²
Cane corso	Neuronal ceroid lipofuscinosis	<i>PPT1</i> ⁵³
Tibetan terrier	Progressive retinal atrophy	<i>FAM161A</i> ²³⁵
Shiba inu	GM2 gangliosidosis	<i>HEXB</i> *
Dachshund	Methemoglobinemia	<i>CYB5R3</i> *
Mixed breed	Methemoglobinemia	<i>CYB5R3</i> *
Ns duck tolling retriever	Degenerative encephalopathy	<i>RB1CC1</i> *
Mixed breed	Beta-mannosidosis	<i>MANBA</i> *

*manuscripts on different stages of completion

There have been significant differences in the quality of sequence data generated at different points in time that result from changes in sequencing chemistry and instrumentation. Prior to 2016, the University of Missouri sequences were generated using an Illumina HiSeq 2500 which could generate up to 1 TB of data in six days when using the high-output mode and 300 GB when using the rapid run mode. The paired read lengths of HiSeq 2500 varied between 2x 50bp to 2x 125bp, and the cost per sample was ~\$5,000 for a 30X average coverage of a ~2.6GB genome. From 2016, the Illumina HiSeq X Ten series became available for use with non-human samples. This new technology is almost four times faster it can produce 3.6 TB in six days at a reduced cost of ~\$2,000 per sample (30X coverage). HiSeq X Ten improved the sequencing chemistry and technology, decreasing the error rates and slightly increased the read lengths (2x 150bp). There are also differences in the library preparation methodologies between the older and more current sequences. The earlier sequences (2010-2013) used PCR-based libraries with fragment lengths of 300 bp and 400 bp. Between 2013 and 2015, kits became available to produce PCR-free libraries with fragment lengths of 350 bp and 550 bp.

PCR-based library preparation generally consists of fragmenting the DNA molecules, fusing the fragments to adapters, followed by amplification. The DNA fragmentation step were achieved by either mechanical force (sonication, nebulization or acoustic shearing) or by enzymatic digestion (non-specific endonuclease cocktails). The ends of the fragments were enzymatically repaired and the adapter sequences were ligated to each end. The fragments of the desired

size can be selected by either gel extraction, or by using solid-phase reversible immobilization (SPRI) beads. The amplification to enrich the product that has the adapters ligated to both ends is then achieved by PCR cycles and the sample is ready for the sequencing by synthesis.^{236,237} PCR-free protocols require a higher concentration of input DNA than the PCR methodology, and use kits such as the Illumina TruSeq DNA PCR-free kit that include DNA fragmentation, fragment size selection and final library quantification in their protocols.

Slightly different PCR-free library preparations were used for the newer HiSeq X Ten sequences, where the fragment lengths are limited to 350bp or 450 bp, because of the physical limitations of the new, bead-dependent ordered array flow-cells that are used in this technology. The newer flow cells have much more surface area for cluster generation, which is how the HiSeq X Ten can generate so much more data than the earlier machines. Each flow cell and sequencing technology supports an optimal insert size. Smaller insert sizes amplify more efficiently, however there is a risk of generating a lower number of reads and coverage, and if a much larger insert size is sequenced, there is a possibility of overloading the clustering and bridge amplification step, causing a high number of duplicates, sequencing overlapping, cross-contamination of multiplexed samples and index hopping (mislabeling of reads).

As mentioned in Chapter 1, library preparation is an important step that can introduce biases to the sequences and variation calling. The difference was very noticeable especially between the PCR libraries and the PCR-free libraries. The PCR-based libraries had many sequencing gaps and inconsistent coverage (zero

to 33X) along the genome, especially along high GC% and other repetitive regions. High GC% regions require a higher melting point, due to the strong hydrogen bonds, and often times have low primer specificity. In addition, PCR artifacts, including amplification bias, mispriming events, and higher number of duplicate sequences, resulted in many false positive variants that could not be later verified with Sanger sequencing. Many of the false positives were within the first exons, which can be explained by the high GC% content of those regions. Sometimes those false positive variants and lower coverage occurred within functionally relevant genes for the diseases that were being researched at the time, and were considered plausible candidates until disproved by Sanger sequencing. Since the lab started to use PCR-free library based sequences, most of the single nucleotide candidate variants reported in the WGS could be later verified through other molecular methods.

Improvements in the computational resources and bioinformatics pipelines were also accomplished over time. Initially, the computational resources were more limited and fewer samples could be processed simultaneously. The entire data processing (including trimming, QC, alignment and variant calling) with the older available computational resources could take from one and a half week to two weeks per sample. Because the canine lab is not the only one that generates a high number of WGS at the University of Missouri, it could take a few months before a variant report was available and annotated. A "Biocomputer cluster" was developed as a part of the 2016 Cyberinfrastructure Plan Update at the University of Missouri (<https://mospace.umsystem.edu/xmlui/handle/10355/48334>). The

cluster is currently available and consists of 37 Dell R630 Broadwell nodes with 28 cores, 512 GB of RAM, and 3TB of local SSD storage. The expanded HPC resources have the potential to process over 30 individuals per day with the current QC and variant calling pipeline.

Several algorithms were developed overtime to analyze sequencing data, and they can be broadly classified in different categories and subcategories. For example, preprocessing and quality control software include adapter trimmers, duplicate read removal tools, error correction, variant recalibration, and other algorithms. Data analysis algorithms include read aligners, variant callers, copy number variant callers, annotators and visualization tools. One of the reasons for the “forest of software” in the bioinformatics field is because many laboratories develop their own algorithms optimized for their own data and biological question. Some of those software packages are no longer maintained or updated, hence checking the dates of the latest update and frequencies of the updates is important. For this reason, there are efforts to curate the available and up to date bioinformatics software in one place, such as (OMICtools)²³⁸. Best practices and user friendly pipelines for whole genome sequence analysis were developed by different institutions and companies, with the goal of helping biologists with little or no computational background. Some developed freely available software, such as the Genome Analysis Toolkit (Broad institute)²³⁹ and Galaxy²⁴⁰, and some others offer commercially available software, such as Softgenetics NextGeneTM, Geneious²⁴¹, CLCbio Genomics Workbench²⁴², Golden Helix SNP and Variation SuiteTM, among others. Some of these software attempt to integrate algorithms pertaining to each

different WGS analysis step into just one user friendly interface, such as the one from Softgenetics NextGene™.

The bioinformatics pipeline used within the Johnson lab from 2010 until early 2015 was mostly done by in-house perl scripts for adapter trimming and quality control, and the NextGene software was used for aligning the reads to a reference sequence, variant calling, annotation and visualization. The variants were filtered and stored in a PostgreSQL database. The downside of the NextGene software, other than the cost of the software license, is that the file format of the output is specific to NextGene and extra steps that can add a few days to the processing time are required to convert the files to a more universal format, such as BAM, if the use of different analysis software is desired. In my experience, the file conversion sometimes resulted in corrupted and unusable BAM files. The time used for the troubleshooting such file conversion errors could be used for other more productive analysis.

As increasingly more individual sequences become available, a joint analysis is recommended. Joint analysis uses the evidence from multiple samples to determine the genotypes of all samples, calling the variants occurring in sites with lower coverage with a much higher accuracy and sensitivity than individual variant calling.²³⁹ However, joint analysis also introduced a problem called the “N+1” problem.²³⁹ Joint analysis requires that all samples are run together in one batch because not all >100 samples were generated at the same time, whenever a new sample was introduced, the whole batch variant calling needed to be re-run from scratch. As expected, the more samples that are added to the batch, the more

computationally intensive it becomes to generate the variant calls, resulting in a scalability problem. These problems were addressed by the genome analysis toolkit (GATK) best practices pipeline with the introduction of the GVCF (genomic variant call files) workflow²³⁹. The GVCF files are similar to regular variant call files (VCF). The difference is that GVCFs contain a record of every position in the genome, or the selected interval, regardless of whether a variant was detected at that site or not. For example, when a newer sample with a certain number of “new” variants and “new” coordinates that were not previously called on the older samples is added to the cohort, the GVCF of the older samples already contains information about each sample’s probable genotype in those “new” coordinates, unlike the regular VCF, thus, GVCFs save time and computational power when doing the joint analysis. Since individual GVCF files are generated and stored for each sample as a first step, the joint analysis can be done at any time, using any cohort of samples as desired.

Once the variant calling is done, the next step for detecting causal mutations is variant filtering and annotation. There are two main annotation sources, NCBI (Refseq) and Ensembl. Both sources use different procedures for their gene predictions: Refseq is a collection of non-redundant curated mRNA models, and Ensembl is a database that contains gene models from multiple sources mapped into the reference genome. Therefore, filtering variants based on those two annotation sources can result in very different variant classifications. From the end of 2015, our lab purchased a lab license for the Golden Helix SNP and Variation Suite™ (SVS), and this software has a user-friendly interface for annotating and

filtering variants. The variant call files of 120 dogs were annotated with the SVS software using both, Ensembl and RefSeq sources. The total number of variants in this cohort that were classified as potentially functional (variants that are near or within exons and are non-synonymous) was 115,614. The variants that were common to both NCBI and Ensembl annotation sources was 50,599 (43.8%) however 21,715 (18.8%) were only detected by the Ensembl annotation and 43,300 (47.5%) were detected only by the NCBI annotation. In chapter 5, the causal mutation on *HEXB* was almost missed because only the Ensembl annotation was interrogated at the time, and the causal deletion was placed in the “unknown classification” bin based on that annotation. Using both annotation sources is important, and even then the causal variants could still be misclassified and be filtered out of the final variant reports, as seen on Table 2. The two instances in Table 2 where the mutations were not present on either annotation report are the mutations on the *FAN1* and *PPT1* genes. Both of those mutations were detected through the visual inspection of the alignments using a genome browser, like IGV.^{204,243} Those genes in particular were visually inspected because of previous genome mapping studies (*FAN1*) or because of known previous associations between human orthologs and human diseases with similar phenotypes (*PPT1*). The *FAN1* mutation was probably absent from the reports not because of an annotation error, but because the causal mutation is a large deletion and those types of variants are not reported by the regular variant calling algorithms. Large insertions, deletions, inversions and duplications require specific structural variant (SV) callers that are not yet well established in our pipeline. The

PPT1 mutation is a splicing mutation, and splicing mutations have major annotation discrepancy problems. In order to predict splice site effects, an accurate exon-intron boundary must be annotated in a gene, and complete transcript information is helpful, if not essential. Some genes have multiple isoforms and it is difficult to know which isoform is active in the cell type of interest. Also, there are overlapping genes that might share an exon, or the variant falls in the exon of one of the genes and in the intron, splice-site, promoter or regulatory region for the other gene. Even genes with only one isoform might harbor variants with more than one consequence, and all of those issues must be considered when evaluating candidate mutations.²⁴⁴

Table 5. List of genes that were successfully associated to a disease phenotype by Dr. GS Johnson's lab, and which annotation source correctly predicted the variant effect and included the variant in the final mutation report.

Causal Gene	Ensembl	NCBI	Both	None*
<i>SERAC1a</i>			X	
<i>FAN1</i>				X
<i>PIGN</i>			X	
<i>KCNJ10</i>		X		
<i>SERAC1b</i>			X	
<i>SAG</i>			X	
<i>MFSD8</i>			X	
<i>RBM20</i>			X	
<i>CLN8</i>			X	
<i>RAB3GAP1</i>			X	
<i>STK11IP</i>			X	
<i>CLN5a</i>			X	
<i>CLN5b</i>			X	
<i>PPT1</i>				X
<i>FAM161A</i>			X	
<i>HEXB</i>		X		
<i>CYB5R3a</i>	X			
<i>CYB5R3b</i>	X			
<i>RB1CC1</i>			X	

So far our successes in finding disease-causing mutations were done either by direct candidate gene scanning or rare variant filtering. The direct candidate gene scanning approach is used when there is previous knowledge of genes that are

associated with a particular disease phenotype. This methodology was previously mentioned and described with the *FAN1*, *CLN5* (Chapter 3) and *PPT1* (Chapter 4) examples. Visually inspecting a candidate gene approach does not necessarily require a variant report. The visualization of the reads aligned to the reference sequence is done simply by loading a BAM file, the reference sequence (FASTA file) and the annotation sources (GFF or other file formats) into a genome browser. Any potential deleterious mutations near or within the exons of the biologically known candidate genes are then noted and further tested for phenotypic association. When there are hundreds of possible candidate genes, all the variants occurring within those genes can be filtered in the variant reports, rather than visually inspecting thousands of exons. This approach could be used to find potential risk factors for epilepsy, for example. Sometimes the mutation is within a gene that was never associated to the specific disease phenotype before, which was the case for the *PIGN* mutation (Chapter 2). In this case, one affected individual had 418 unique mutations in comparison to 100 control dogs. Since none of the 302 genes harboring these mutations had a clear biological association to the paroxysmal dyskinesia, a second affected dog was sequenced and the only variant that was common to both affected dogs and was not present in the other 100 control dogs was the *PIGN*:c.398C>T substitution. Unfortunately, the approach of sequencing more than one affected dog and filtering the rare variants has not yet been successful for the identification of the causal mutations of some other diseases, such as the standard poodle polymicrogyria (PMG), border terrier canine epileptoid cramping syndrome (CECS), border terrier dysmyelinogenesis,

gordon setter “darned unnamed new genetic disease” (DUNGd), Jack Russel terrier neonatal granulo-prival ataxia and bouvier des Fladres glaucoma. There are many possible reasons why the approach has not yet worked for those cases. First, unlike the paroxysmal dyskinesia cases where only one mutation was unique to both affected individuals within the cohort, these other cases have several unique variants, where most have no obvious biological correlation, and are yet to be tested for phenotype-genotype association. Also, there is a chance that the chosen affected samples do not have the same disease and are “phenocopies” Furthermore, as mentioned before, the variant reports do not contain information about structural and copy number variants. There could be possible annotation errors or incomplete annotation, especially involving the regulatory regions within introns or even intergenic. Other types of variants that are being underexplored at the moment are mutations in CpG islands that could cause loss of imprinting, or other epigenetic effects.

Some of those diseases also have GWAS data generated as an attempt to narrow down the locus of the causal mutation. A few candidate variants within the associated regions are currently under investigation for many of these diseases, including a structural change within an intron that could potentially disrupt a splice site on *FIG4* in Gordon Setters with DUNGd. Recently the lab acquired the Agena MassArray system that could potentially genotype up to 30 variants at once. However, so far our experience with designing synergetic and consistent assays has been disappointing. Once the technical difficulties of dealing with the

equipment and technology are addressed, we might be able to “kiss multiple frogs at once” and more efficiently discover disease-associated genotypes.

There are other ways to analyze and filter variants that can potentially lead to the discovery of new genotype-phenotype relationships, such as incidental findings and “reverse genetics”. Incidental findings happen in situations where you find a deleterious mutation in a gene known to cause a phenotype that is different than the disease in the original research plan. For example, a dog that was sequenced because of a heart defect that also has a deleterious mutation in a gene that is known to cause a late onset eye disease. Reverse genetics can be defined as the process of determining which phenotypes arise as a result of a particular variant.²⁴⁵ Reverse genetics using WGS could be accomplished by identifying individuals that are heterozygous for a deleterious mutation that could potentially cause disease in the homozygous state, and testing multiple individuals of the same family or breed until a homozygous mutant can be found to verify that the expected particular disease phenotype occurs in this individual. Those two methodologies can be hard to achieve because they may require a very detailed clinical follow up and the cooperation of the sample providers is required, which unfortunately is not always achievable.

Collaboration with veterinary clinicians and an accurate characterization of the disease is very important for different steps of the research, including the process for choosing which samples to sequence. In chapter 5, the *HEXB* mutation causing GM2 gangliosidosis could have been discovered by directly sequencing the *HEXB* gene and save sequencing money, if an enzymatic assay had been performed by

the clinicians. However, this example also showed that WGS can be useful for personalized veterinary medicine as it can correctly provide a diagnosis.

During my graduate studies I have analyzed 8 different GWAS and tested over 35 different candidate variants that were not associated with the expected disease phenotype. Of the 8 GWAS, one was done in collaboration with the University of Pennsylvania, where I identified a locus on chromosome 9 associated to increased susceptibility with *Mycobacterium avium* complex (MAC) in Miniature Standard Schnauzers. Recently, researchers at University of Philadelphia identified the phenotype-associated mutation, a homozygous codon deletion in the *CARD9* gene that is present within the locus and was highlighted through my analysis. More recently, I detected a single nucleotide variant in the *MANBA* gene in a mixed breed dog that was suspected of having a lysosomal storage disease, consistent with a diagnosis of β -mannosidosis. Enzymatic analysis has confirmed this diagnosis.

With the current and future improvements optimization of the variant discovery pipeline, the mutation analysis will become faster and easier. Traits that are not Mendelian are still very challenging and could also explain why we only have a ~30% discovery success rate. The current optimized variant calling algorithms are not able to detect large deletions, insertions, inversions, insertions of LINEs or SINEs and copy number variations, which can also potentially explain some of the disease causing variations. Also, it still hard to determine the impact of intronic, 5'-UTR, 3'-UTR and other regulatory variants with the current canine genome annotation, and as discussed earlier, we are perhaps still missing many causal

variants due to annotation errors, reference sequence gaps or other data generation or analysis biases. Sequencing technologies with longer reads are already available but too expensive for many routine uses. In the future they will probably cost much less and they will add a lot of information concerning structural differences among individual and cohort samples. There is a current international and multi-collaborative effort to test many of the open source structural variant (SV) and copy number variant (CNV) calling software packages in over 500 canine genomes. The aim is to better characterize the canine genome and potentially detect disease causing variations that are not reported by the GATK or platypus variant callers. In order to identify potential risk factors for complex traits such as epilepsy, different strategies will be required as the risk alleles might be common in the general population, but will only have effect in individuals that have a burden of other risk alleles.

There are still many improvements to be made, but WGS analysis is without doubt a very efficient methodology for detecting disease-causing mutations. The potential conjunction of WGS with other molecular methods such as ATAC-seq for identifying active chromatin and regulatory portions of the genome in the cell type of interest, and CRISPR/Cas9 for assisting in gene function discovery, is very exciting. Improvements in sequencing technology and analytic tools will lead to interesting discoveries that help our best friends to be healthier and happier.

References

1. Ember CR, Ember M. Encyclopedia of medical anthropology : health and illness in the world's cultures. New York: Kluwer Academic/Plenum Publishers; 2004.
2. Lamarck JBPAdMd. Histoire naturelle des animaux sans vertèbres : présentant les caractères généraux et particuliers de ces animaux ... : précédée d'une introduction offrant la détermination des caractères essentiels de l'animal, sa distinction du végétal et des autres corps naturels, enfin, l'exposition des principes fondamentaux de la zoologie. Paris: Verdière; 1815.
3. Wang Y, Liu H, Sun Z. Lamarck rises from his grave: parental environment-induced epigenetic inheritance in model organisms and humans. Biol Rev Camb Philos Soc 2017.
4. Darwin C. On the origin of species by means of natural selection, or, The preservation of favoured races in the struggle for life, 5th thousand. ed. London,: J. Murray; 1860;ix, 1 , 502 p.
5. Mendel G, Fisher RA. Experiments in plant hybridisation. Edinburgh,: Oliver & Boyd; 1965;ix, 95 p.
6. Gayon J. From Mendel to epigenetics: History of genetics. C R Biol 2016;339:225-230.
7. Dahm R. Friedrich Miescher and the discovery of DNA. Dev Biol 2005;278:274-288.
8. Miescher F. Ueber die chemische Zusammensetzung der Eiterzellen. Medicinisch-chemische Untersuchungen 1871;4:441-460.
9. Mccarty M. A Retrospective Look at the Discovery of the Genetic Role of DNA. Faseb J 1994;8:889-890.
10. Pray L. Discovery of DNA structure and function: Watson and Crick. Nature Education 2008;1:100.
11. Watson JD, Crick FH. The structure of DNA. Cold Spring Harb Symp Quant Biol 1953;18:123-131.

12. Levene PA. On the Chemistry of the Chromatin Substance of the Nerve Cell. *J Med Res* 1903;10:204-211.
13. Chargaff E, Vischer E. Nucleoproteins, nucleic acids, and related substances. *Annu Rev Biochem* 1948;17:201-226.
14. Chargaff E, Magasanik B, et al. The nucleotide composition of ribonucleic acids. *J Am Chem Soc* 1949;71:1513.
15. RajBhandary UL, Kohrer C. Early days of tRNA research: discovery, function, purification and sequence analysis. *J Biosci* 2006;31:439-451.
16. Sanger F, Donelson JE, Coulson AR, et al. Use of DNA polymerase I primed by a synthetic oligonucleotide to determine a nucleotide sequence in phage ϕ 1 DNA. *Proc Natl Acad Sci U S A* 1973;70:1209-1213.
17. Maxam AM, Gilbert W. A new method for sequencing DNA. *Proc Natl Acad Sci U S A* 1977;74:560-564.
18. Kulski JK. Next-Generation Sequencing — An Overview of the History, Tools, and “Omic” Applications. 2016.
19. Saiki RK, Gelfand DH, Stoffel S, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988;239:487-491.
20. Barba M, Czosnek H, Hadidi A. Historical perspective, development and applications of next-generation sequencing in plant virology. *Viruses* 2014;6:106-136.
21. Franca LT, Carrilho E, Kist TB. A review of DNA sequencing techniques. *Q Rev Biophys* 2002;35:169-200.
22. Heather JM, Chain B. The sequence of sequencers: The history of sequencing DNA. *Genomics* 2016;107:1-8.

23. Dewey FE, Pan S, Wheeler MT, et al. DNA sequencing: clinical applications of new DNA sequencing technologies. *Circulation* 2012;125:931-944.
24. Consortium IHGS. Initial sequencing and analysis of the human genome. *Nature* 2001;409.
25. Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. *Science* 2001;291:1304-1351.
26. Waterston RH, Lander ES, Sulston JE. On the sequencing of the human genome. *Proc Natl Acad Sci U S A* 2002;99:3712-3716.
27. Koepfli KP, Paten B, Genome KCoS, et al. The Genome 10K Project: a way forward. *Annu Rev Anim Biosci* 2015;3:57-111.
28. Genome KCoS. Genome 10K: a proposal to obtain whole-genome sequence for 10,000 vertebrate species. *J Hered* 2009;100:659-674.
29. Stephens ZD, Lee SY, Faghri F, et al. Big Data: Astronomical or Genomical? *PLoS Biol* 2015;13:e1002195.
30. van Steenbeek FG, Hytonen MK, Leegwater PA, et al. The canine era: the rise of a biomedical model. *Anim Genet* 2016;47:519-527.
31. Wang GD, Zhai W, Yang HC, et al. The genomics of selection in dogs and the parallel evolution between dogs and humans. *Nat Commun* 2013;4:1860.
32. Schoenebeck JJ, Ostrander EA. The genetics of canine skull shape variation. *Genetics* 2013;193:317-325.
33. Boyko AR. The domestic dog: man's best friend in the genomic era. *Genome Biol* 2011;12:216.
34. Freedman AH, Lohmueller KE, Wayne RK. Evolutionary History, Selective Sweeps, and Deleterious Variation in the Dog. *Annual Review of Ecology, Evolution, and Systematics* 2016;47:73-96.

35. Fan Z, Silva P, Gronau I, et al. Worldwide patterns of genomic variation and admixture in gray wolves. *Genome Res* 2016;26:163-173.
36. Skoglund P, Ersmark E, Palkopoulou E, et al. Ancient wolf genome reveals an early divergence of domestic dog ancestors and admixture into high-latitude breeds. *Curr Biol* 2015;25:1515-1519.
37. Reiter T, Jagoda E, Capellini TD. Dietary Variation and Evolution of Gene Copy Number among Dog Breeds. *PLoS One* 2016;11:e0148899.
38. vonHoldt BM, Pollinger JP, Earl DA, et al. A genome-wide perspective on the evolutionary history of enigmatic wolf-like canids. *Genome Res* 2011;21:1294-1305.
39. Lindblad-Toh K, Wade CM, Mikkelsen TS, et al. Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature* 2005;438:803-819.
40. Kirkness EF, Bafna V, Halpern AL, et al. The dog genome: survey sequencing and comparative analysis. *Science* 2003;301:1898-1903.
41. Ostrander EA, Lindblad-Toh K, Lander ES, et al. Sequencing the Genome of the Domestic Dog *Canis familiaris*. Grant proposal.
42. Batzoglou S, Jaffe DB, Stanley K, et al. ARACHNE: a whole-genome shotgun assembler. *Genome Res* 2002;12:177-189.
43. Hoepfner MP, Lundquist A, Pirun M, et al. An improved canine genome and a comprehensive catalogue of coding genes and non-coding transcripts. *PLoS One* 2014;9:e91172.
44. Mellersh CS, Langston AA, Acland GM, et al. A linkage map of the canine genome. *Genomics* 1997;46:326-336.
45. Yuzbasiyan-Gurkan V, Blanton SH, Cao Y, et al. Linkage of a microsatellite marker to the canine copper toxicosis locus in Bedlington terriers. *Am J Vet Res* 1997;58:23-27.

46. Lohi H, Young EJ, Fitzmaurice SN, et al. Expanded repeat in canine epilepsy. *Science* 2005;307:81.
47. Voorbij AM, van Steenbeek FG, Vos-Loohuis M, et al. A contracted DNA repeat in LHX3 intron 5 is associated with aberrant splicing and pituitary dwarfism in German shepherd dogs. *PLoS One* 2011;6:e27940.
48. Karlsson EK, Baranowska I, Wade CM, et al. Efficient mapping of mendelian traits in dogs through genome-wide association. *Nat Genet* 2007;39:1321-1328.
49. Cadieu E, Neff MW, Quignon P, et al. Coat variation in the domestic dog is governed by variants in three genes. *Science* 2009;326:150-153.
50. Awano T, Johnson GS, Wade CM, et al. Genome-wide association analysis reveals a SOD1 mutation in canine degenerative myelopathy that resembles amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* 2009;106:2794-2799.
51. Faller KM, Bras J, Sharpe SJ, et al. The Chihuahua dog: A new animal model for neuronal ceroid lipofuscinosis CLN7 disease? *J Neurosci Res* 2016;94:339-347.
52. Guo J, Johnson GS, Brown HA, et al. A CLN8 nonsense mutation in the whole genome sequence of a mixed breed dog with neuronal ceroid lipofuscinosis and Australian Shepherd ancestry. *Mol Genet Metab* 2014;112:302-309.
53. Kolichski A, Barnes Heller HL, Arnold S, et al. Homozygous PPT1 Splice Donor Mutation in a Cane Corso Dog With Neuronal Ceroid Lipofuscinosis. *J Vet Intern Med* 2017;31:149-157.
54. Hirz M, Drogemuller M, Schanzer A, et al. Neuronal ceroid lipofuscinosis (NCL) is caused by the entire deletion of CLN8 in the Alpenlandische Dachsbracke dog. *Mol Genet Metab* 2017;120:269-277.
55. Niskanen J, Dillard K, Arumilli M, et al. Nonsense variant in COL7A1 causes recessive dystrophic epidermolysis bullosa in Central Asian Shepherd dogs. *PLoS One* 2017;12:e0177527.

56. Bentley DR, Balasubramanian S, Swerdlow HP, et al. Accurate whole human genome sequencing using reversible terminator chemistry. *Nature* 2008;456:53-59.
57. Rothberg JM, Hinz W, Rearick TM, et al. An integrated semiconductor device enabling non-optical genome sequencing. *Nature* 2011;475:348-352.
58. Eid J, Fehr A, Gray J, et al. Real-time DNA sequencing from single polymerase molecules. *Science* 2009;323:133-138.
59. Mardis ER. A decade's perspective on DNA sequencing technology. *Nature* 2011;470:198-203.
60. Illumina. An introduction to Next-Generation Sequencing Technology. www.illumina.com/technology/next-generation-sequencing.html 2016.
61. Horner DS, Pavesi G, Castrignano T, et al. Bioinformatics approaches for genomics and post genomics applications of next-generation sequencing. *Brief Bioinform* 2010;11:181-197.
62. Doyle K. DNA Quantitation in Next-Generation Sequencing Library Workflows. Promega Corporation Website <http://www.promega.com/resources/pubhub/dna-quantitation-in-next-generation-sequencing-library-workflows/> 2015.
63. Aird D, Ross MG, Chen WS, et al. Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries. *Genome Biol* 2011;12:R18.
64. McCall CM, Mosier S, Thiess M, et al. False positives in multiplex PCR-based next-generation sequencing have unique signatures. *J Mol Diagn* 2014;16:541-549.
65. Ross MG, Russ C, Costello M, et al. Characterizing and measuring bias in sequence data. *Genome Biol* 2013;14:R51.
66. Illumina. Quality Scores for Next-Generation Sequencing: Assessing sequencing accuracy using Phred quality scoring. Technical Note: Sequencing 2011.

67. Endrullat C, Glokler J, Franke P, et al. Standardization and quality management in next-generation sequencing. *Appl Transl Genom* 2016;10:2-9.
68. Taylor JC, Martin HC, Lise S, et al. Factors influencing success of clinical genome sequencing across a broad spectrum of disorders. *Nat Genet* 2015;47:717-726.
69. Choi M, Scholl UI, Ji W, et al. Genetic diagnosis by whole exome capture and massively parallel DNA sequencing. *Proc Natl Acad Sci U S A* 2009;106:19096-19101.
70. Bolze A, Byun M, McDonald D, et al. Whole-exome-sequencing-based discovery of human FADD deficiency. *Am J Hum Genet* 2010;87:873-881.
71. Worthey EA, Mayer AN, Syverson GD, et al. Making a definitive diagnosis: successful clinical application of whole exome sequencing in a child with intractable inflammatory bowel disease. *Genet Med* 2011;13:255-262.
72. Manolio TA, Collins FS, Cox NJ, et al. Finding the missing heritability of complex diseases. *Nature* 2009;461:747-753.
73. Manolio TA. Genomewide association studies and assessment of the risk of disease. *N Engl J Med* 2010;363:166-176.
74. Kingsmore SF, Saunders CJ. Deep sequencing of patient genomes for disease diagnosis: when will it become routine? *Sci Transl Med* 2011;3:87ps23.
75. Green ED, Guyer MS, National Human Genome Research I. Charting a course for genomic medicine from base pairs to bedside. *Nature* 2011;470:204-213.
76. Roychowdhury S, Iyer MK, Robinson DR, et al. Personalized oncology through integrative high-throughput sequencing: a pilot study. *Sci Transl Med* 2011;3:111ra121.
77. Pleasance ED, Cheetham RK, Stephens PJ, et al. A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature* 2010;463:191-196.

78. Snyder TM, Khush KK, Valantine HA, et al. Universal noninvasive detection of solid organ transplant rejection. *Proc Natl Acad Sci U S A* 2011;108:6229-6234.
79. Palomaki GE, Kloza EM, Lambert-Messerlian GM, et al. DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. *Genet Med* 2011;13:913-920.
80. Kolicheski AL, Johnson GS, Mhlanga-Mutangadura T, et al. A homozygous PIGN missense mutation in Soft-Coated Wheaten Terriers with a canine paroxysmal dyskinesia. *Neurogenetics* 2017;18:39-47.
81. Livak KJ. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genetic Analysis: Biomolecular Engineering* 1999;14:143-149.
82. Kolicheski A, Johnson GS, O'Brien DP, et al. Australian Cattle Dogs with Neuronal Ceroid Lipofuscinosis are Homozygous for a CLN5 Nonsense Mutation Previously Identified in Border Collies. *J Vet Intern Med* 2016;30:1149-1158.
83. Demirkiran M, Jankovic J. Paroxysmal dyskinesias: clinical features and classification. *Ann Neurol* 1995;38:571-579.
84. Bhatia KP. Paroxysmal dyskinesias. *Mov Disord* 2011;26:1157-1165.
85. Jankovic J, Demirkiran M. Classification of paroxysmal dyskinesias and ataxias. *Adv Neurol* 2002;89:387-400.
86. Waln O, Jankovic J. Paroxysmal movement disorders. *Neurol Clin* 2015;33:137-152.
87. Erro R, Sheerin UM, Bhatia KP. Paroxysmal dyskinesias revisited: a review of 500 genetically proven cases and a new classification. *Mov Disord* 2014;29:1108-1116.
88. Bruno MK, Lee HY, Auburger GW, et al. Genotype-phenotype correlation of paroxysmal nonkinesigenic dyskinesia. *Neurology* 2007;68:1782-1789.

89. Bruno MK, Hallett M, Gwinn-Hardy K, et al. Clinical evaluation of idiopathic paroxysmal kinesigenic dyskinesia: new diagnostic criteria. *Neurology* 2004;63:2280-2287.
90. Blakeley J, Jankovic J. Secondary paroxysmal dyskinesias. *Mov Disord* 2002;17:726-734.
91. Chen WJ, Lin Y, Xiong ZQ, et al. Exome sequencing identifies truncating mutations in PRRT2 that cause paroxysmal kinesigenic dyskinesia. *Nat Genet* 2011;43:1252-1255.
92. Rainier S, Thomas D, Tokarz D, et al. Myofibrillogenesis regulator 1 gene mutations cause paroxysmal dystonic choreoathetosis. *Arch Neurol* 2004;61:1025-1029.
93. Suls A, Dedeken P, Goffin K, et al. Paroxysmal exercise-induced dyskinesia and epilepsy is due to mutations in SLC2A1, encoding the glucose transporter GLUT1. *Brain* 2008;131:1831-1844.
94. Wang JL, Cao L, Li XH, et al. Identification of PRRT2 as the causative gene of paroxysmal kinesigenic dyskinesias. *Brain* 2011;134:3493-3501.
95. Lee HY, Xu Y, Huang Y, et al. The gene for paroxysmal non-kinesigenic dyskinesia encodes an enzyme in a stress response pathway. *Hum Mol Genet* 2004;13:3161-3170.
96. Brockmann K. The expanding phenotype of GLUT1-deficiency syndrome. *Brain Dev* 2009;31:545-552.
97. Anand G, Padaniya A, Hanrahan D, et al. Milder phenotypes of glucose transporter type 1 deficiency syndrome. *Dev Med Child Neurol* 2011;53:664-668.
98. Schneider SA, Paisan-Ruiz C, Garcia-Gorostiaga I, et al. GLUT1 gene mutations cause sporadic paroxysmal exercise-induced dyskinesias. *Mov Disord* 2009;24:1684-1688.
99. Weber YG, Storch A, Wuttke TV, et al. GLUT1 mutations are a cause of paroxysmal exertion-induced dyskinesias and induce hemolytic anemia by a cation leak. *J Clin Invest* 2008;118:2157-2168.

100. Li M, Niu F, Zhu X, et al. PRRT2 Mutant Leads to Dysfunction of Glutamate Signaling. *Int J Mol Sci* 2015;16:9134-9151.
101. Nobile C, Striano P. PRRT2: a major cause of infantile epilepsy and other paroxysmal disorders of childhood. *Prog Brain Res* 2014;213:141-158.
102. Shen Y, Ge WP, Li Y, et al. Protein mutated in paroxysmal dyskinesia interacts with the active zone protein RIM and suppresses synaptic vesicle exocytosis. *Proc Natl Acad Sci U S A* 2015;112:2935-2941.
103. Du W, Bautista JF, Yang H, et al. Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat Genet* 2005;37:733-738.
104. Castiglioni C, Verrigni D, Okuma C, et al. Pyruvate dehydrogenase deficiency presenting as isolated paroxysmal exercise induced dystonia successfully reversed with thiamine supplementation. Case report and mini-review. *Eur J Paediatr Neurol* 2015;19:497-503.
105. Urkasemsin G, Olby NJ. Canine paroxysmal movement disorders. *Vet Clin North Am Small Anim Pract* 2014;44:1091-1102.
106. Penderis J, Franklin RJ. Dyskinesia in an adult bichon frise. *J Small Anim Pract* 2001;42:24-25.
107. Black V, Garosi L, Lowrie M, et al. Phenotypic characterisation of canine epileptoid cramping syndrome in the Border terrier. *J Small Anim Pract* 2014;55:102-107.
108. Herrtage ME, Palmer AC. Episodic falling in the cavalier King Charles spaniel. *Vet Rec* 1983;112:458-459.
109. Packer RA, Patterson EE, Taylor JF, et al. Characterization and mode of inheritance of a paroxysmal dyskinesia in Chinook dogs. *J Vet Intern Med* 2010;24:1305-1313.
110. Wolf M, Bruehschwein A, Sauter-Louis C, et al. An inherited episodic head tremor syndrome in Doberman pinscher dogs. *Mov Disord* 2011;26:2381-2386.

111. Guevar J, De Decker S, Van Ham LM, et al. Idiopathic head tremor in English bulldogs. *Mov Disord* 2014;29:191-194.
112. Meyers KM, Lund JE, Padgett G, et al. Hyperkinetic episodes in Scottish Terrier dogs. *J Am Vet Med Assoc* 1969;155:129-133.
113. Shelton GD. Muscle pain, cramps and hypertonicity. *Vet Clin North Am Small Anim Pract* 2004;34:1483-1496.
114. Forman OP, Penderis J, Hartley C, et al. Parallel mapping and simultaneous sequencing reveals deletions in BCAN and FAM83H associated with discrete inherited disorders in a domestic dog breed. *PLoS Genet* 2012;8:e1002462.
115. Gill JL, Tsai KL, Krey C, et al. A canine BCAN microdeletion associated with episodic falling syndrome. *Neurobiol Dis* 2012;45:130-136.
116. Gilliam D, O'Brien DP, Coates JR, et al. A homozygous KCNJ10 mutation in Jack Russell Terriers and related breeds with spinocerebellar ataxia with myokymia, seizures, or both. *J Vet Intern Med* 2014;28:871-877.
117. Guo J, O'Brien DP, Mhlanga-Mutangadura T, et al. A rare homozygous MFSD8 single-base-pair deletion and frameshift in the whole genome sequence of a Chinese Crested dog with neuronal ceroid lipofuscinosis. *BMC Vet Res* 2015;10:960.
118. Ohba C, Okamoto N, Murakami Y, et al. PIGN mutations cause congenital anomalies, developmental delay, hypotonia, epilepsy, and progressive cerebellar atrophy. *Neurogenetics* 2014;15:85-92.
119. Kinoshita T. Biosynthesis and deficiencies of glycosylphosphatidylinositol. *Proceedings of the Japan Academy, Series B* 2014;90:130-143.
120. Sanger TD, Delgado MR, Gaebler-Spira D, et al. Classification and definition of disorders causing hypertonia in childhood. *Pediatrics* 2003;111:e89-97.

121. Sanger TD, Chen D, Fehlings DL, et al. Definition and classification of hyperkinetic movements in childhood. *Mov Disord* 2010;25:1538-1549.
122. Bhatia KP, Soland VL, Bhatt MH, et al. Paroxysmal exercise-induced dystonia: eight new sporadic cases and a review of the literature. *Mov Disord* 1997;12:1007-1012.
123. Hooser SB, VR B. Methylxanthine (chocolate and caffeine toxicosis). . Philadelphia: 1986;pp. 191-192.
124. Kinoshita T, Fujita M, Maeda Y. Biosynthesis, remodelling and functions of mammalian GPI-anchored proteins: recent progress. *J Biochem* 2008;144:287-294.
125. Orlean P, Menon AK. Thematic review series: lipid posttranslational modifications. GPI anchoring of protein in yeast and mammalian cells, or: how we learned to stop worrying and love glycopospholipids. *J Lipid Res* 2007;48:993-1011.
126. Gaynor EC, Mondesert G, Grimme SJ, et al. MCD4 encodes a conserved endoplasmic reticulum membrane protein essential for glycosylphosphatidylinositol anchor synthesis in yeast. *Mol Biol Cell* 1999;10:627-648.
127. Hong Y, Maeda Y, Watanabe R, et al. Pig-n, a mammalian homologue of yeast Mcd4p, is involved in transferring phosphoethanolamine to the first mannose of the glycosylphosphatidylinositol. *J Biol Chem* 1999;274:35099-35106.
128. Vainauskas S, Menon AK. Ethanolamine phosphate linked to the first mannose residue of glycosylphosphatidylinositol (GPI) lipids is a major feature of the GPI structure that is recognized by human GPI transamidase. *J Biol Chem* 2006;281:38358-38364.
129. Khayat M, Tilghman JM, Chervinsky I, et al. A PIGN mutation responsible for multiple congenital anomalies-hypotonia-seizures syndrome 1 (MCAHS1) in an Israeli-Arab family. *Am J Med Genet A* 2016;170A:176-182.

130. Nakagawa T, Taniguchi-Ikeda M, Murakami Y, et al. A novel PIGN mutation and prenatal diagnosis of inherited glycosylphosphatidylinositol deficiency. *Am J Med Genet A* 2016;170A:183-188.
131. Maydan G, Noyman I, Har-Zahav A, et al. Multiple congenital anomalies-hypotonia-seizures syndrome is caused by a mutation in PIGN. *J Med Genet* 2011;48:383-389.
132. McKean DM, Niswander L. Defects in GPI biosynthesis perturb Cripto signaling during forebrain development in two new mouse models of holoprosencephaly. *Biol Open* 2012;1:874-883.
133. Brady PD, Moerman P, De Catte L, et al. Exome sequencing identifies a recessive PIGN splice site mutation as a cause of syndromic congenital diaphragmatic hernia. *Eur J Med Genet* 2014;57:487-493.
134. Couser NL, Masood MM, Strande NT, et al. The phenotype of multiple congenital anomalies-hypotonia-seizures syndrome 1: report and review. *Am J Med Genet A* 2015;167A:2176-2181.
135. Fleming L, Lemmon M, Beck N, et al. Genotype-phenotype correlation of congenital anomalies in multiple congenital anomalies hypotonia seizures syndrome (MCAHS1)/PIGN-related epilepsy. *Am J Med Genet A* 2016;170A:77-86.
136. Groffen AJ, Klapwijk T, van Rootselaar AF, et al. Genetic and phenotypic heterogeneity in sporadic and familial forms of paroxysmal dyskinesia. *J Neurol* 2013;260:93-99.
137. Mole SE, Cotman SL. Genetics of the neuronal ceroid lipofuscinoses (Batten disease). *Biochim Biophys Acta* 2015;1852:2237-2241.
138. Jolly RD, Palmer DN. The neuronal ceroid-lipofuscinoses (Batten disease): comparative aspects. *Neuropathol Appl Neurobiol* 1995;21:50-60.

139. Anderson GW, Goebel HH, Simonati A. Human pathology in NCL. *Biochim Biophys Acta* 2013;1832:1807-1826.
140. Sanders DN, Farias FH, Johnson GS, et al. A mutation in canine PPT1 causes early onset neuronal ceroid lipofuscinosis in a Dachshund. *Mol Genet Metab* 2010;100:349-356.
141. Awano T, Katz ML, O'Brien DP, et al. A frame shift mutation in canine TPP1 (the ortholog of human CLN2) in a juvenile Dachshund with neuronal ceroid lipofuscinosis. *Mol Genet Metab* 2006;89:254-260.
142. Melville SA, Wilson CL, Chiang CS, et al. A mutation in canine CLN5 causes neuronal ceroid lipofuscinosis in Border collie dogs. *Genomics* 2005;86:287-294.
143. Gilliam D, Kolicheski A, Johnson GS, et al. Golden Retriever dogs with neuronal ceroid lipofuscinosis have a two-base-pair deletion and frameshift in CLN5. *Mol Genet Metab* 2015;115:101-109.
144. Katz ML, Farias FH, Sanders DN, et al. A missense mutation in canine CLN6 in an Australian shepherd with neuronal ceroid lipofuscinosis. *J Biomed Biotechnol* 2011;2011:198042.
145. Katz ML, Khan S, Awano T, et al. A mutation in the CLN8 gene in English Setter dogs with neuronal ceroid-lipofuscinosis. *Biochem Biophys Res Commun* 2005;327:541-547.
146. Awano T, Katz ML, O'Brien DP, et al. A mutation in the cathepsin D gene (CTSD) in American Bulldogs with neuronal ceroid lipofuscinosis. *Mol Genet Metab* 2006;87:341-348.
147. Farias FH, Zeng R, Johnson GS, et al. A truncating mutation in ATP13A2 is responsible for adult-onset neuronal ceroid lipofuscinosis in Tibetan terriers. *Neurobiol Dis* 2011;42:468-474.
148. Wohlke A, Philipp U, Bock P, et al. A one base pair deletion in the canine ATP13A2 gene causes exon skipping and late-onset neuronal ceroid lipofuscinosis in the Tibetan terrier. *PLoS Genet* 2011;7:e1002304.

149. Cho DY, Leipold HW, Rudolph R. Neuronal ceroidosis (ceroid-lipofuscinosis) in a Blue Heeler dog. *Acta Neuropathol* 1986;69:161-164.
150. Nimmo Wilkie JS, Hudson EB. Neuronal and generalized ceroid-lipofuscinosis in a cocker spaniel. *Vet Pathol* 1982;19:623-628.
151. Goebel HH, Bilzer T, Dahme E, et al. Morphological studies in canine (Dalmatian) neuronal ceroid-lipofuscinosis. *Am J Med Genet Suppl* 1988;5:127-139.
152. Rossmeisl JHJ, Duncan R, Fox J, et al. Neuronal ceroid-lipofuscinosis in a Labrador Retriever. *J Vet Diagn Invest* 2003;15:457-460.
153. Jolly RD, Sutton RH, Smith RI, et al. Ceroid-lipofuscinosis in miniature Schnauzer dogs. *Aust Vet J* 1997;75:67.
154. Narfstrom K, Wrigstad A. Clinical, electrophysiological, and morphological findings in a case of neuronal ceroid lipofuscinosis in The Polish Owczarek Nizinny (PON) dog. *Vet Q* 1995;17:46.
155. Appleby EC, Longstaffe JA. Ceroid-lipofuscinosis in two saluki dogs. *JComp Path* 1982;92.
156. Zeng R, Coates JR, Johnson GC, et al. Breed distribution of SOD1 alleles previously associated with canine degenerative myelopathy. *J Vet Intern Med* 2014;28:515-521.
157. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009;25:1754-1760.
158. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 2009;25:2078-2079.
159. Rimmer A, Phan H, Mathieson I, et al. Integrating mapping-, assembly- and haplotype-based approaches for calling variants in clinical sequencing applications. *Nat Genet* 2014;46:912-918.
160. Rama Rao KV, Kielian T. Astrocytes and lysosomal storage diseases. *Neuroscience* 2016;323:195-206.

161. Wood PA, Sisk DB, Styer E, et al. Animal model: ceroidosis (ceroid-lipofuscinosis) in Australian cattle dogs. *Am J Med Genet* 1987;26:891-898.
162. Sisk DB, Levesque DC, Wood PA, et al. Clinical and pathologic features of ceroid lipofuscinosis in two Australian cattle dogs. *J Am Vet Med Assoc* 1990;197:361-364.
163. Taylor RM, Farrow BR. Ceroid lipofuscinosis in the border collie dog: retinal lesions in an animal model of juvenile Batten disease. *Am J Med Genet* 1992;42:622-627.
164. Taylor RM, Farrow BR. Ceroid-lipofuscinosis in border collie dogs. *Acta Neuropathol* 1988;75:627-631.
165. Studdert VP, Mitten RW. Clinical features of ceroid lipofuscinosis in border collie dogs. *Aust Vet J* 1991;68:137-140.
166. Franks JN, Dewey CW, Walker MA, et al. Computed tomographic findings of ceroid lipofuscinosis in a dog. *J Am Anim Hosp Assoc* 1999;35:430-435.
167. Mizukami K, Kawamichi T, Koie H, et al. Neuronal ceroid lipofuscinosis in Border Collie dogs in Japan: clinical and molecular epidemiological study (2000-2011). *ScientificWorldJournal* 2012;2012:383174.
168. Mizukami K, Chang HS, Yabuki A, et al. Novel rapid genotyping assays for neuronal ceroid lipofuscinosis in Border Collie dogs and high frequency of the mutant allele in Japan. *J Vet Diagn Invest* 2011;23:1131-1139.
169. Savukoski M, Klockars T, Holmberg V, et al. CLN5, a novel gene encoding a putative transmembrane protein mutated in Finnish variant late infantile neuronal ceroid lipofuscinosis. *Nat Genet* 1998;19:286-288.
170. Xin W, Mullen TE, Kiely R, et al. CLN5 mutations are frequent in juvenile and late-onset non-Finnish patients with NCL. *Neurology* 2010;74:565-571.

171. Kousi M, Lehesjoki AE, Mole SE. Update of the mutation spectrum and clinical correlations of over 360 mutations in eight genes that underlie the neuronal ceroid lipofuscinoses. *Hum Mutat* 2012;33:42-63.
172. Mancini C, Nassani S, Guo Y, et al. Adult-onset autosomal recessive ataxia associated with neuronal ceroid lipofuscinosis type 5 gene (CLN5) mutations. *J Neurol* 2015;262:173-178.
173. Carcel-Trullols J, Kovacs AD, Pearce DA. Cell biology of the NCL proteins: What they do and don't do. *Biochim Biophys Acta* 2015;1852:2242-2255.
174. Kopra O, Vesa J, von Schantz C, et al. A mouse model for Finnish variant late infantile neuronal ceroid lipofuscinosis, CLN5, reveals neuropathology associated with early aging. *Hum Mol Genet* 2004;13:2893-2906.
175. Frugier T, Mitchell NL, Tammen I, et al. A new large animal model of CLN5 neuronal ceroid lipofuscinosis in Borderdale sheep is caused by a nucleotide substitution at a consensus splice site (c.571+1G>A) leading to excision of exon 3. *Neurobiol Dis* 2008;29:306-315.
176. Amorim IS, Mitchell NL, Palmer DN, et al. Molecular neuropathology of the synapse in sheep with CLN5 Batten disease. *Brain Behav* 2015;5:e00401.
177. Hughes SM, Hope KM, Xu JB, et al. Inhibition of storage pathology in prenatal CLN5-deficient sheep neural cultures by lentiviral gene therapy. *Neurobiol Dis* 2014;62:543-550.
178. Perentos N, Martins AQ, Watson TC, et al. Translational neurophysiology in sheep: measuring sleep and neurological dysfunction in CLN5 Batten disease affected sheep. *Brain* 2015;138:862-874.
179. Palmer DN, Neverman NJ, Chen JZ, et al. Recent studies of ovine neuronal ceroid lipofuscinoses from BARN, the Batten Animal Research Network. *Biochim Biophys Acta* 2015;1852:2279-2286.

180. Bradbury AM, Gurda BL, Casal ML, et al. A review of gene therapy in canine and feline models of lysosomal storage disorders. *Hum Gene Ther Clin Dev* 2015;26:27-37.
181. Katz ML, Coates JR, Sibigroth CM, et al. Enzyme replacement therapy attenuates disease progression in a canine model of late-infantile neuronal ceroid lipofuscinosis (CLN2 disease). *J Neurosci Res* 2014;92:1591-1598.
182. Vuilleminot BR, Kennedy D, Cooper JD, et al. Nonclinical evaluation of CNS-administered TPP1 enzyme replacement in canine CLN2 neuronal ceroid lipofuscinosis. *Mol Genet Metab* 2015;114:281-293.
183. Katz ML, Tecedor L, Chen Y, et al. AAV gene transfer delays disease onset in a TPP1-deficient canine model of the late infantile form of Batten disease. *Sci Transl Med* 2015;7:313ra180.
184. Weleber RG, Gupta N, Trzupek KM, et al. Electroretinographic and clinicopathologic correlations of retinal dysfunction in infantile neuronal ceroid lipofuscinosis (infantile Batten disease). *Mol Genet Metab* 2004;83:128-137.
185. Burset M, Seledtsov IA, Solovyev VV. Analysis of canonical and non-canonical splice sites in mammalian genomes. *Nucleic Acids Res* 2000;28:4364-4375.
186. Krawczak M, Thomas NS, Hundrieser B, et al. Single base-pair substitutions in exon-intron junctions of human genes: nature, distribution, and consequences for mRNA splicing. *Hum Mutat* 2007;28:150-158.
187. Baralle D, Baralle M. Splicing in action: assessing disease causing sequence changes. *J Med Genet* 2005;42:737-748.
188. Yeo G, Burge CB. Maximum entropy modeling of short sequence motifs with applications to RNA splicing signals. *J Comput Biol* 2004;11:377-394.

189. Popp MW, Maquat LE. Organizing principles of mammalian nonsense-mediated mRNA decay. *Annu Rev Genet* 2013;47:139-165.
190. Vesa J, Hellsten E, Verkruyse LA, et al. Mutations in the palmitoyl protein thioesterase gene causing infantile neuronal ceroid lipofuscinosis. *Nature* 1995;376:584-587.
191. Gupta P, Soyombo AA, Atashband A, et al. Disruption of PPT1 or PPT2 causes neuronal ceroid lipofuscinosis in knockout mice. *Proc Natl Acad Sci U S A* 2001;98:13566-13571.
192. Jalanko A, Vesa J, Manninen T, et al. Mice with Ppt1Deltaex4 mutation replicate the INCL phenotype and show an inflammation-associated loss of interneurons. *Neurobiol Dis* 2005;18:226-241.
193. Bouchelion A, Zhang Z, Li Y, et al. Mice homozygous for c.451C>T mutation in Cln1 gene recapitulate INCL phenotype. *Ann Clin Transl Neurol* 2014;1:1006-1023.
194. Miller JN, Kovacs AD, Pearce DA. The novel Cln1(R151X) mouse model of infantile neuronal ceroid lipofuscinosis (INCL) for testing nonsense suppression therapy. *Hum Mol Genet* 2015;24:185-196.
195. Sarkar C, Chandra G, Peng S, et al. Neuroprotection and lifespan extension in Ppt1(-/-) mice by NtBuHA: therapeutic implications for INCL. *Nat Neurosci* 2013;16:1608-1617.
196. Finn R, Kovacs AD, Pearce DA. Treatment of the Ppt1(-/-) mouse model of infantile neuronal ceroid lipofuscinosis with the N-methyl-D-aspartate (NMDA) receptor antagonist memantine. *J Child Neurol* 2013;28:1159-1168.
197. Lu JY, Nelvagal HR, Wang L, et al. Intrathecal enzyme replacement therapy improves motor function and survival in a preclinical mouse model of infantile neuronal ceroid lipofuscinosis. *Mol Genet Metab* 2015;116:98-105.

198. Thada V, Miller JN, Kovacs AD, et al. Tissue-specific variation in nonsense mutant transcript level and drug-induced read-through efficiency in the Cln1(R151X) mouse model of INCL. *J Cell Mol Med* 2016;20:381-385.
199. Tracy CJ, Sanders DN, Bryan JN, et al. Intravitreal Implantation of Genetically Modified Autologous Bone Marrow-Derived Stem Cells for Treating Retinal Disorders. *Adv Exp Med Biol* 2016;854:571-577.
200. Griffey MA, Wozniak D, Wong M, et al. CNS-directed AAV2-mediated gene therapy ameliorates functional deficits in a murine model of infantile neuronal ceroid lipofuscinosis. *Mol Ther* 2006;13:538-547.
201. Aungaroon G, Hallinan B, Jain P, et al. Correlation Among Genotype, Phenotype, and Histology in Neuronal Ceroid Lipofuscinoses: An Individual Patient Data Meta-Analysis. *Pediatr Neurol* 2016.
202. Evans J, Katz ML, Levesque D, et al. A variant form of neuronal ceroid lipofuscinosis in American bulldogs. *J Vet Intern Med* 2005;19:44-51.
203. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;30:2114-2120.
204. Robinson JT, Thorvaldsdottir H, Winckler W, et al. Integrative genomics viewer. *Nat Biotechnol* 2011;29:24-26.
205. Mowjoodi A, Paton TA, Scherer SW. Discrimination of SNPs in GC-rich regions using a modified hydrolysis probe chemistry protocol. *Biotechniques* 2014;57:313-316.
206. Yamato O, Endoh D, Kobayashi A, et al. A novel mutation in the gene for canine acid beta-galactosidase that causes GM1-gangliosidosis in Shiba dogs. *J Inherit Metab Dis* 2002;25:525-526.

207. Lawson CA, Martin DR. Animal models of GM2 gangliosidosis: utility and limitations. *Appl Clin Genet* 2016;9:111-120.
208. Sandhoff K, Harzer K. Gangliosides and gangliosidoses: principles of molecular and metabolic pathogenesis. *J Neurosci* 2013;33:10195-10208.
209. Sanders DN, Zeng R, Wenger DA, et al. GM2 gangliosidosis associated with a HEXA missense mutation in Japanese Chin dogs: a potential model for Tay Sachs disease. *Mol Genet Metab* 2013;108:70-75.
210. Brownstein S, Carpenter S, Polomeno RC, et al. Sandhoff's disease (GM2 gangliosidosis type 2). Histopathology and ultrastructure of the eye. *Arch Ophthalmol* 1980;98:1089-1097.
211. Lowden JA, Callahan JW, Gravel RA, et al. Type 2 GM1 gangliosidosis with long survival and neuronal ceroid lipofuscinosis. *Neurology* 1981;31:719-724.
212. Fontaine G, Resibois A, Tondeur M, et al. Gangliosidosis with total hexosaminidase deficiency: clinical, biochemical and ultrastructural studies and comparison with conventional cases of Tay-Sachs disease. *Acta Neuropathol* 1973;23:118-132.
213. Suzuki K. Neuropathology of late onset gangliosidoses. A review. *Dev Neurosci* 1991;13:205-210.
214. Oldfors A, Sourander P. Storage of lipofuscin in neurons in mucopolysaccharidosis. Report on a case of Sanfilippo's syndrome with histochemical and electron-microscopic findings. *Acta Neuropathol* 1981;54:287-292.
215. Radke J, Stenzel W, Goebel HH. Human NCL Neuropathology. *Biochim Biophys Acta* 2015;1852:2262-2266.
216. Wisniewski K, Rudelli R, Laure-Kamionowska M, et al. Sanfilippo disease, type A with some features of ceroid lipofuscinosis. *Neuropediatrics* 1985;16:98-105.

217. Damme M, Stroobants S, Walkley SU, et al. Cerebellar alterations and gait defects as therapeutic outcome measures for enzyme replacement therapy in alpha-mannosidosis. *J Neuropathol Exp Neurol* 2011;70:83-94.
218. Siso S, Navarro C, Hanzlicek D, et al. Adult onset thalamocerebellar degeneration in dogs associated to neuronal storage of ceroid lipopigment. *Acta Neuropathol* 2004;108:386-392.
219. Abitbol M, Thibaud JL, Olby NJ, et al. A canine Arylsulfatase G (ARSG) mutation leading to a sulfatase deficiency is associated with neuronal ceroid lipofuscinosis. *Proc Natl Acad Sci U S A* 2010;107:14775-14780.
220. Kowalewski B, Lamanna WC, Lawrence R, et al. Arylsulfatase G inactivation causes loss of heparan sulfate 3-O-sulfatase activity and mucopolysaccharidosis in mice. *Proc Natl Acad Sci U S A* 2012;109:10310-10315.
221. Yamato O, Ochiai K, Masuoka Y, et al. GM1 gangliosidosis in shiba dogs. *Vet Rec* 2000;146:493-496.
222. Yamato O, Masuoka Y, Yonemura M, et al. Clinical and clinico-pathologic characteristics of Shiba dogs with a deficiency of lysosomal acid beta-galactosidase: a canine model of human GM1 gangliosidosis. *J Vet Med Sci* 2003;65:213-217.
223. Karbe E, Schiefer B. Familial amaurotic idiocy in male German shorthair pointers. *Pathol Vet* 1967;4:223-232.
224. Cummings JF, Wood PA, Walkley SU, et al. GM2 gangliosidosis in a Japanese spaniel. *Acta Neuropathol* 1985;67:247-253.
225. Yamato O, Matsuki N, Satoh H, et al. Sandhoff disease in a golden retriever dog. *J Inherit Metab Dis* 2002;25:319-320.
226. Tamura S, Tamura Y, Uchida K, et al. GM2 gangliosidosis variant O (Sandhoff-like disease) in a family of toy poodles. *J Vet Intern Med* 2010;24:1013-1019.

227. Kohyama M, Yabuki A, Kawasaki Y, et al. GM2 Gangliosidosis Variant 0 (Sandhoff Disease) in a Mixed-Breed Dog. *J Am Anim Hosp Assoc* 2015;51:396-400.
228. Rahman MM, Chang HS, Mizukami K, et al. A frameshift mutation in the canine HEXB gene in toy poodles with GM2 gangliosidosis variant 0 (Sandhoff disease). *Vet J* 2012;194:412-416.
229. Precone V, Del Monaco V, Esposito MV, et al. Cracking the Code of Human Diseases Using Next-Generation Sequencing: Applications, Challenges, and Perspectives. *Biomed Res Int* 2015;2015:161648.
230. Park ST, Kim J. Trends in Next-Generation Sequencing and a New Era for Whole Genome Sequencing. *Int Neurol J* 2016;20:S76-83.
231. Hall N. It's only human. *Genome Biol* 2014;15:104.
232. Watson M. Illuminating the future of DNA sequencing. *Genome Biol* 2014;15:108.
233. Mhlanga-Mutangadura T, Johnson GS, Schnabel RD, et al. A mutation in the Warburg syndrome gene, RAB3GAP1, causes a similar syndrome with polyneuropathy and neuronal vacuolation in Black Russian Terrier dogs. *Neurobiol Dis* 2016;86:75-85.
234. Mhlanga-Mutangadura T, Johnson GS, Ashwini A, et al. A Homozygous RAB3GAP1:c.743delC Mutation in Rottweilers with Neuronal Vacuolation and Spinocerebellar Degeneration. *J Vet Intern Med* 2016;30:813-818.
235. Downs LM, Mellersh CS. An Intronic SINE insertion in FAM161A that causes exon-skipping is associated with progressive retinal atrophy in Tibetan Spaniels and Tibetan Terriers. *PLoS One* 2014;9:e93990.
236. van Dijk EL, Jaszczyszyn Y, Thermes C. Library preparation methods for next-generation sequencing: tone down the bias. *Exp Cell Res* 2014;322:12-20.
237. Head SR, Komori HK, LaMere SA, et al. Library construction for next-generation sequencing: overviews and challenges. *Biotechniques* 2014;56:61-64, 66, 68, passim.

238. Henry VJ, Bandrowski AE, Pepin AS, et al. OMICtools: an informative directory for multi-omic data analysis. Database (Oxford) 2014;2014.
239. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010;20:1297-1303.
240. Afgan E, Baker D, van den Beek M, et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. Nucleic Acids Res 2016;44:W3-W10.
241. Kears M, Moir R, Wilson A, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 2012;28:1647-1649.
242. CLC Genomics Workbench 9.5.3. In. <https://www.qiagenbioinformatics.com/>.
243. Thorvaldsdottir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinform 2013;14:178-192.
244. McCarthy DJ, Humburg P, Kanapin A, et al. Choice of transcripts and software has a large effect on variant annotation. Genome Med 2014;6:26.
245. Griffiths AJF MJ, Suzuki DT, et al. . An Introduction to Genetic Analysis., 7th edition ed. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK21843/>; New York: W. H. Freeman; 2000.

VITA

Ana Leticia Kolicheski was born in 1985 in the city Curitiba, capital of the state of Parana (PR) in Brazil. She obtained her Bachelor's in Veterinary Medicine at the Universidade Federal do Parana (UFPR) in December 2009, Curitiba, PR, and her two Master's degree in Animal Breeding and Genetics were completed at Wageningen University (WU), Wageningen, in The Netherlands, and at the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden in 2012. She started her PhD studies at the University of Missouri in February of 2013 and completed her PhD in July of 2017.