Minnesota Leonberger Health Project:
Update, Plans and Request
December 2021

Update

You are all now very familiar with the LPN1, LPN2, LPPN3, and LEMP mutation discoveries and the genetic testing we have offered. The LPN1 (D/N & D/D genotypes), LPN2 (D/N & D/D genotypes) and LPPN3 (D/D genotype) mutations combined account for almost half of all historical cases of laryngeal paralysis (LP) in our biobank.

Sample Database and Genetic Testing. A major fraction of Katie Minor's time is spent on maintaining the sample and medical record database and providing an on-demand genetic testing service. Our DNA repository and medical record database, combined with the University of Bern, now contains > 10,300 Leonberger DNA samples. It is worth noting that over the past several years the University of Minnesota has performed testing on > 2700 dogs. LHF support allowed us to provide ~ 300 LPPN3 results to owners with LP affected dogs at no charge. Also, with the launch of the LPPN3 test last November Katie has reported out ~1100 results to ~550 different owners. Each of these requires at least 2 emails, and sometimes several more!

	2017	2018	2019	2020	2021
Dogs Tested	1192	265	226	547	485
Note	LEMP launch			LPPN3 launch (Nov)	

In addition, we have provided Leonberger DNA samples to the University of Bern for glaucoma and renal dysplasia projects and are in the process of sending 25 samples to Brian Catchpole at Oxford University for Anal Furunculosis (fistula) research. We have also sent imputed SNP genotype data from thousands of Leonbergers to Hannes Lohi at the University of Helsinki for their cardiac study.

Further, by integrating publicly available data from the OFA/CHIC database into our system, we have been able to match their medical information data with DNA we have available for research, including: >1200 thyroid results, >1400 hip and elbow scores, >600 normal cardiac evaluations, and >750 cerf eye exams with information on persistent pupillary membrane, entropion, distichiasis and cataracts.

In summary, supporting Katie 's efforts in performing this Leonberger sample database maintenance and testing service on demand allows us to be more responsive to other researchers, breeder's and owner's needs.

Additional genetic causes of LPPN. We believe that the majority of the currently unexplained LPPN diagnoses are likely comprised of a collection of single gene (monogenic) and multiple gene (polygenic) causes. These as yet genetically-unascribed cases have an average age of onset 5.5 years (range 10 weeks – 13 years) and have a higher proportion of males (75%) than females (25%). Approximately half of them have an age of onset of < 6 years, which are more likely to have a more severe form of disease in which a simple genetic cause may be responsible.

The Universities of Minnesota and Bern now have a combined whole genome sequence database from 67 Leonbergers, including 49 individuals showing variable neurologic signs (40 of which are as yet genetically un-ascribed). Analysis of whole genome sequences from these Leonbergers with neurological disease identified several mutations in genes affecting nerve function that appeared to be unique to Leonbergers, or at least have extremely low frequencies in other breeds. During the past year our Bern collaborators genotyped 12 of the most promising variants in a population of 96 Leonberger LPN cases that were negative for the LPN1, LPN2 and LPN3 mutations, along with 96 controls over 8 years of age. The results failed to provide evidence of a significant association of any of these variants with a diagnosis of a neurological disorder (i.e., similar allele frequencies were found in the cases and controls). However, there is a significant bias towards males as cases in this population (see Table, below).

Sex Distribution of Genetically as yet Un-ascribed LPPN Cases

	Controls	Cases	Controls	Cases
Males	221	289	38.04%	75.65%
Females	360	93	61.96%	24.35%
Grand Total	581	382	100.00%	100.00%

We have initiated a new effort to attempt to identify novel mutations in LPPN Leonbergers by returning to the genome wide association approach which we used previously for identifying the LPN1, LPN2, LPPN3, and LEMP mutations. We recently received SNP genotyping array data for 58 new LPN cases and 33 male controls. This brings the total for our new analyses to 345 cases (266 male and 79 female), and 394 controls (189 male and 205 female). Controls dogs must be at least 8 years of age at the time of last reported normal health questionnaire (average in our GWAS is 9.5 years). Among these genetically un-ascribed cases, we have identified 164 dogs with an age of onset < 6 years.

Osteosarcoma. The Universities of Minnesota and Bern have also collected medical and genetic data on osteosarcoma with the goal of increasing our knowledge of the genetic factors that contribute to developing this form of bone cancer in Leonbergers. We shared our data with Dr Kerstin Lindblad-Toh and her research group at Uppsala University feeling that these leaders in canine cancer genetics would be best equipped to perform the next phase of the analysis. However, in late summer, after discussions with Kerstin, the Bern and Minnesota groups agreed to take over the osteosarcoma data analysis. We are now pleased to report that our analysis has resulted in a peer-reviewed publication: https://doi.org/10.3390/genes12121964.

Briefly, the breed prevalence of osteosarcoma in our database is approximately 20%. We performed several genomic analyses using genome-wide high-density imputed SNP genotype data from 273 Leonberger osteosarcoma cases with a median age of 8.1 years (range of 3.1-13.5) and 365 controls older than 8 years. First, we were able to estimate the heritability of osteosarcoma in Leonbergers to be 20.6 %. 2,563 SNPs across the genome accounted for nearly all the heritability, but with each SNP contributing just a small fraction to the total heritability. These results demonstrate that osteosarcoma in Leonbergers is a moderately heritable complex polygenic disease (i.e., many different gene mutations and environmental factors contribute).

We then performed an association analysis to attempt to localize major chromosomal loci conferring susceptibility. This analysis revealed a significant association with SNPs on canine chromosome 11, mirroring previous findings in other dog breeds, such as the greyhound, that also show an elevated risk for osteosarcoma. Subsequently, we repeated the analysis with a subset of younger cases (7 years or less, n=77) and older controls (10 years or more, n=184) that revealed the strongest association at the same chromosome 11 locus as when using all animals, with additional suggestive loci on chromosomes 4, 20, 22, 29, and 37.

The region of significance on chromosome 11 encompasses approximately 2 million bp of DNA. The SNP genotype data further revealed a variety of versions (i.e., haplotypes) of the contiguous run of DNA in this region. But, a haplotype containing the most significant SNPs was present at a significantly higher frequency in cases compared to controls (0.53 versus 0.38; chi-square P-value 9.02×10^{-8}). And the distribution of osteosarcoma cases carrying 0, 1 or 2 copies of the associated haplotype (20.5%, 52.7%, 26.7%) was significantly different than the control dogs (37.8%, 48.2%, 14.0%, respectively.

This chromosome 11 segment contains the *CDKN2A* and *CDKN2B* genes which have been previously described in osteosarcoma-affected greyhounds, as well as in histiocytic sarcoma-affected Bernese mountain dogs, flat-coated retrievers and rottweilers. Our findings confirm a complex genetic basis of OSA, moderate heritability, and the crucial role of the *CDKN2A/B* locus leading to strong cancer predisposition.

Hypothyroidism. Last year we described a preliminary, but significant locus of interest for hypothyroidism on chromosome 36, obtained with 52 cases and 65 controls. Over the past year, with the owner-reported OFA/CHIC data included we have increased this SNP genotyped cohort to 4 autoimmune thyroiditis, 125 owner reported hypothyroid cases, and 203 dogs with a "normal" result reported on OFA. We also have an additional 102 DNA samples from affected dogs that have not been SNP genotyped.

Planned work

Continue database maintenance and testing service and Leonberger community contacts and outreach. We expect the need for genetic testing and community outreach to continue, with possible impacts of any new mutations that might be discovered.

Advancing genetics of novel neurological disorders. We plan to finish our genome-wide DNA marker studies using the almost 400 cases described above that are negative for the LPN1, LPN2 and LPPN3 mutations, and long-lived dogs that show no signs of neuro disease as controls. If one or major chromosomal loci are revealed, we will use whole genome sequences to identify variants and hopefully be able to work this up to identifying the causal mutation(s) and add to the genetic testing portfolio.

Osteosarcoma. We will attempt to more accurately define the genetic variants contributing the major risk for developing osteosarcoma, recognizing that it will likely constitute a real challenge, as with many other cancers it is likely that regulatory, non-coding variants underlie the increased risk for cancer development. Given a high proportion of control dogs that contain the chromosome 11 risk haplotype this small set of markers, on their own, may not be sufficient for use in long term breeding strategies aimed at reducing the odds of dogs developing OSA. Nevertheless, an ultimate goal could be a multi-marker genetic assay containing many hundreds of SNPs that contribute to the heritability that could assess the likelihood of an individual dog developing osteosarcoma. Such a test could conceivably be used for preemptive diagnostic strategies.

Hypothyroidism. We will be analyzing this SNP genotyping data this upcoming year. Again, depending on the DNA marker analysis, the ultimate goal could be a genetic assay to assess the likelihood of an individual dog developing hypothyroidism, that could be used for earlier therapeutic intervention to help control adverse effects of the disease.

Addison's disease. We have identified 18 Addison's disease cases in our database and have genotyped 15 on SNP arrays but have not yet identified a major locus. We plan to redo the GWAS while removing the hypothyroid cases from the analysis, as both of these conditions are thought to have an autoimmune origin.