Analysis of copy number variation in dogs implicates genomic structural variation in the development of anterior cruciate ligament rupture

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Abstract

Anterior cruciate ligament (ACL) rupture is an important condition of the human knee. Second ruptures are common and societal costs are substantial. Canine cranial cruciate ligament (CCL) rupture closely models the human disease. CCL rupture is common in the Labrador Retriever (5.79% prevalence), ~100-fold more prevalent than in humans. Labrador Retriever CCL rupture is a polygenic complex disease, based on genome-wide association study (GWAS) of single nucleotide polymorphism (SNP) markers. Dissection of genetic variation in complex traits can be enhanced by studying structural variation, including copy number variants (CNVs). Dogs are an ideal model for CNV research because of reduced genetic variability within breeds and extensive phenotypic diversity across breeds. We studied the genetic etiology of CCL rupture by association analysis of CNV regions (CNVRs) using 110 case and 164 control Labrador Retrievers. CNVs were called from SNPs using three different programs (PennCNV, CNVPartition, and QuantiSNP). After quality control, CNV calls were combined to create CNVRs using ParseCNV and an association analysis was performed. We found no strong effect CNVRs but found 46 small effect (max(T) permutation \( P < 0.05 \)) CCL rupture associated CNVRs in 22 autosomes; 25 were deletions and 21 were duplications. Of the 46 CCL rupture associated CNVRs, we identified 39 unique regions. Thirty four were identified by a single calling algorithm, 3 were identified by two calling algorithms, and 2 were identified by all three algorithms. For 42 of the associated CNVRs, frequency in the population was \(<10\%\) while 4 occurred at a frequency in the population ranging from 10–25\%. Average CNVR length was 198,872bp and CNVRs covered 0.11 to 0.15\% of the genome. All CNVRs were associated with case status. CNVRs did not overlap previous canine CCL rupture risk loci identified by GWAS. Associated CNVRs contained 152 annotated genes; 12 CNVRs did not have genes mapped to CanFam3.1. Using pathway analysis, a cluster of 19 homeobox domain transcript regulator genes was associated with CCL rupture (\( P = 6.6E-13 \)). This gene cluster influences cranial-caudal body pattern.
formation during embryonic limb development. Clustered genes were found in 3 CNVRs on chromosome 14 (HoxA), 28 (NKX6-2), and 36 (HoxD). When analysis was limited to deletion CNVRs, the association was strengthened ($P = 8.7E-16$). This study suggests a component of the polygenic risk of CCL rupture in Labrador Retrievers is associated with small effect CNVs and may include aspects of stifle morphology regulated by homeobox domain transcript regulator genes.

## Introduction

Anterior cruciate ligament (ACL) rupture is a common and severe condition of the human knee that is prevalent in individuals who participate in sports [1]. The incidence of ACL rupture per 100,000 person years is estimated at 77–130, with an ACL reconstruction incidence of 43.5 in the USA [2, 3]. Societal costs are substantial, even with the preferred cost-effective treatment of ACL reconstruction [1]. Knee osteoarthritis (OA) is prevalent after ACL rupture, and radiographic progression of OA occurs over time [4]. If ACL rupture is combined with other knee injuries, prevalence of OA increases, and symptoms are worse [4]. A non-contact valgus hyperextension mechanism explains most ACL ruptures [5, 6]. There is a high risk of second ACL rupture by rupture of the contralateral ACL or rupture of an intra-articular graft [7].

ACL rupture is a complex disease determined with genetic and environmental risk [8, 9]. Preemptive identification of at-risk individuals, particularly in athletes, would be advantageous because of the high morbidity and societal cost associated with the condition. Modification of environmental factors could help reduce ACL rupture prevalence [10]. Woman are predisposed to ACL rupture [11]. Family history influences risk of ACL rupture, as an individual with ACL rupture is twice as likely to have a relative with ACL rupture [12]. Candidate gene studies have shown that risk of ACL rupture is influenced by a number of genetic variants, principally genes that influence ligament matrix homeostasis [8, 9]. However, past research provides conflicting data with various limitations in study design leading to risk of bias [8]. A genome-wide association study (GWAS) has been performed in a human sample population of 102,979 with 598 ACL rupture individuals. No single nucleotide polymorphisms (SNPs) were found to meet genome-wide significance for association with affected individuals [13]. In the domestic dog (Canis lupus familiaris), cranial cruciate ligament (CCL) rupture in the stifle is a common condition which has been studied using genome-wide association [14]. Human anatomic terminology (i.e.: ACL, knee, anterior-posterior) will be used going forward for consistency with other canine model papers.

ACL rupture is a common and debilitating condition in many breeds of dog [15] and is typically a non-contact injury [16]. While no animal model can perfectly mirror the biomechanics of the human knee, the canine knee joint has been established as a model for human knee pathology for several decades [17]. Although dogs have a quadrupedal gait, ACL rupture in the two species has many shared clinical features, such as a non-contact mechanism [5, 16], high risk of contralateral rupture [18, 19], bilateral knee OA [18, 20], risk effects from sex steroids [11, 16], shared candidate genes [8, 9, 21], and a shared environment, suggesting that genomic discovery studies in the dog model are highly relevant to human ACL rupture. Similar to humans, progressive ACL fiber tearing develops in the presence of knee synovitis in dogs [20, 22]. Decreased knee laxity associated with low estrogen/progesterone in the follicular phase of the menstrual cycle in human females [11] and effects from ovariohysterectomy in female...
dogs [16] affect ACL rupture risk. Epidemiological risk factors for canine ACL rupture include breed, neutering of both male and female dogs, age, obesity, conformation, and knee synovitis [16, 23–25]. Dog breed is the most important risk factor for disease initiation, as ACL rupture prevalence varies considerably amongst different breeds [15]. Prevalence in the Labrador Retriever is high at 5.79%, while prevalence in a low-risk breed, such as the Greyhound, is low at 0.5% [15]. Pedigree studies have estimated narrow sense additive genetic heritability at 0.27 in the Newfoundland and 0.28 in the Boxer [26, 27]. GWAS in dogs confirmed ACL rupture as a complex polygenic disease with loci on chromosomes 1, 3, and 33 in the Newfoundland [28].

Discovery of complex disease-associated allelic variants is enabled by GWAS. However, disease-associated variants identified by GWAS typically account for small increases in risk when the disease is polygenic. Large scale structural and organizational changes in chromosomes have an important role in genetic susceptibility to common complex disease and often provide a substrate for evolutionary change with the creation of new genes [29, 30]. Copy number variants (CNVs) are large segments of DNA ranging from kilobases (kb) to several megabases (Mb) in length that vary in copy number, when compared to a reference genome [31]. Some disease-associated variants, including CNVs, may go undetected by SNP array GWAS due to small effects, gene-gene interaction, or low probe coverage [32]. Due to challenges detecting multibase variants with SNP markers, SNP array GWAS can be limited at detecting disease-associated structural variations [33]. Dissection of genetic variation in complex traits can be enhanced by studying CNVs [33, 34]. CNVs are extensive throughout the human and canine genomes. There are 100-fold more base pairs affected by CNVs than by SNPs [31]. CNVs can have important biological effects by directly altering gene expression or affecting gene regulation [30].

The dog is an ideal model species for CNV studies [31, 34]. The evolutionary history of domestication and breed development is a unique example of intense artificial selection. For hundreds to thousands of years, dogs have been non-randomly bred, creating bottlenecks and strong selection for specific behavioral and physical characteristics [35]. Dogs are one of the most phenotypically diverse mammals. Studies have scanned the whole genome of several dog breeds looking for breed-specific CNVs that contribute to within-breed patterns of phenotypic variation. For example, a high-density (probe spacing ~1kb) array was used to genotype 61 dogs, 6 of which were Labrador Retrievers, screening the whole canine genome for breed specific CNVs [36]. Labradors were reported to have an average of 179 CNVs per individual, with the mean length of 90.9kb [36]. Another study genotyped 351 dogs using the Illumina CanineHD BeadChip genotyping array (170K SNPs, probe spacing ~13kb) validating CNV detection from commercial SNP arrays [37]. Both studies concluded that breed specific CNVs exist, and many overlap possible disease susceptibility genes [36, 37].

Despite the species-wide diversity in dogs, there is reduced genetic heterogeneity within breeds. Extensive breed-specific genetic patterns explain why many breeds are predisposed to specific diseases. CNVs in the canine genome occupy 4.21% and span over 400 genes [38]. Breed-specific disease phenotypes are known to be influenced by CNVs. A study in Rhodesian and Thai Ridgeback dogs discovered a CNV duplication on chromosome 18 that associated with the dorsal hair ridge [39]. This duplication contains three fibroblast growth factor genes (FGF3, FGF4, and FGF19) that regulate embryonic hair and skin growth. The dorsal hair ridge in Rhodesian and Thai Ridgeback dogs is similar to dermal sinus, a neural-tube defect, in human beings. A genome-wide study of segmental duplications in dogs mapped a CNV affecting the glucokinase (hexokinase 4) regulator (GCKR) gene [38]. In humans, a GWAS identified a significant SNP in GCKR that associates with increased susceptibility to type 2 diabetes [40]. Dogs are an excellent model organism to study genetic diseases because they are affected
by many of the same diseases as humans and reduced within-breed genetic variation enhances the ability to discover underlying genetic mechanisms [32].

To further explore the genetic contribution to ACL rupture we undertook genome-wide detection and analysis of CNVs that associate with ACL rupture in a population of pure-bred Labrador Retrievers. Our aim was to determine whether genomic CNVs form part of the genetic contribution to the polygenic complex trait of ACL rupture. We found 46 small effect CNV regions (CNVRs) associated with case status. Genes contained within these CNVRs were found to cluster to a biologic pathway for homeobox domain transcript regulators that influence anterior-posterior body pattern formation during embryonic limb development.

Materials and methods

Ethics statement

All procedures were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the American Veterinary Medical Association and with approval from the Animal Care Committee of the University of Wisconsin-Madison (protocols V1070 and V005463). Informed consent from each owner was obtained before participation in the study. Animals were recruited from the University of Wisconsin School of Veterinary Medicine.

Dogs

The Labrador Retriever breed was selected because of its high prevalence of ACL rupture [15] and because it is a common breed. All participating dogs were client-owned purebred Labrador Retrievers with four generation pedigree information. Full siblings were excluded from the analysis. A genomic relationship matrix (GRM) was created using SNP data and a singular value decomposition was performed to obtain eigen values and vectors to draw a PCA plot using gaston [41].

An orthopaedic examination to assess knee stability was performed on every dog. Dogs phenotyped as cases had anterior drawer on examination, with ACL rupture confirmed during surgical treatment. Preoperative knee radiographs were collected from affected dogs if available. Control dogs had bilateral lateral knee radiographs taken in a standing position. Lateral knee radiographs were evaluated for the presence of osteophytes and increased soft tissue density in the location of the infrapatellar fat pad due to synovial effusion. These radiographic degenerative changes are characteristic of ACL rupture in dogs [42, 43].

Dogs were considered controls if they were $\geq 8$ years of age, had a normal orthopaedic exam, and normal knee radiographs. Canine ACL rupture is an acquired disease with risk of rupture peaking between ages 2–8 years. Of the 5.79% of Labradors that are affected with cruciate rupture, less than 6% develop ACL rupture after 8 years of age [44].

Radiographic measurement and analysis of proximal tibial slope and relative tibial tuberosity width

Proximal tibial morphology is known to influence risk of ACL rupture in dogs [45, 46]. Therefore, posterior tibial slope (PTS) and relative tibial tuberosity width (rTTW) were measured from lateral radiographs [45–47]. When the entire tibia was not present on the radiographic view, the PTS angle was estimated using an established regression method [47]. Groups were created to calculate median PTS and rTTW for dogs with just the HoxA CNVR deletion, the HoxD CNVR deletion, both HoxA and HoxD CNVR deletions, and all three HoxA, HoxD, and NKC6-2 CNVR deletions. The case and control group median PTS and rTTW excluded any
dog with a \textit{HoxA}, \textit{HoxD}, or \textit{NKX6-2} CNVR deletion. A Mann-Whitney-Wilcoxon test was performed to detect significant differences between case and control group PTS and rTTW.

\textbf{Genotyping}

DNA was isolated from blood (EDTA collection tube) or from a saliva swab (PG-100 oral swabs, DNA Genotek, Ottawa, ON, Canada). Dogs were genotyped using the Illumina CanineHD BeadChip, which contains 173,662 SNP markers with an average probe spacing of 13kb. CanineHD BeadChip SNP locations are annotated on the canine CanFam2.0 genome build.

\textbf{GenomeStudio}

The cluster file encodes normalized expected intensity of green and red fluorescence for all 3 expected genotypes (AA, AB, and BB) for every SNP. The BeadChip manufacturer, Illumina, generates a standard cluster file by genotyping many samples that are representative of the genetic diversity of the species of interest. The standard canine cluster file provided by Illumina was created using a set of 352 dogs from 26 different breeds. It is recommended that a user-generated cluster file is created if the study population is unique and does not fit well to the standard cluster file positions [48]. Creating a user-generated cluster file improves the accuracy of CNV calling and decreases noise. Only 14 of the 352 dogs used to make the standard cluster file were Labrador Retrievers. Consequently, a user-generated cluster file was created using the Gentrain2 cluster generation algorithm. We used 164 phenotype-negative control samples from the purebred Labrador Retriever data set for this file. Newly re-clustered SNPs were zeroed when Call Freq <0.97, Rep Errors >2, P-P-C Errors >2, Cluster Sep ≤0.3, AA R Mean ≤0.2, AB R Mean ≤0.2, BB R Mean ≤0.2, 10% GC Score ≤0.3, Hex Excess >0.2, A/B Freq ≥0.4, AB T Mean <0.2 or >0.8, A/A Freq = 1 and AA T Mean >0.3, A/A Freq = 1 and AA T Dev >0.06, B/B Freq = 1 and BB T Mean <0.7, A/A or B/B Freq = 0 and MAF>0, MAF<0.05 and 0.998>Call Freq>0.99 [48]. A user-generated cluster file was saved and exported from GenomeStudio for all canine autosomes. Chromosomes X and Y were excluded from the analysis.

Data were loaded into GenomeStudio from the raw \texttt{.idat} files, sample sheet, the Illumina provided manifest file for the Canine HD BeadChip, and the user-generated cluster file. Samples with a call rate of <98\% were excluded. SNPs with a genotyping rate of <95\% were excluded. Log R ratio (LRR) and B allele frequency (BAF) values for every autosomal SNP were calculated and exported for each sample. The LRR is a measure of normalized total signal intensity and BAF is a measure of allelic intensity ratios. LRR is expected to equal 0 for every SNP, while BAF is expected to equal 0, 0.5, and 1 [49]. When samples deviate from the expected LRR and BAF, CNVs can be detected.

\textbf{PennCNV}

PennCNV is an algorithm that implements a hidden Markov model to assess the LRR, BAF, the distance between neighboring SNPs, and the population frequency of the B allele (PFB) to detect CNVs [50]. To improve CNV calling, a GC model file was created by calculating the GC content of the 1Mb sequence around each SNP (500kb each side). The PFB file was generated using each SNP marker’s BAF from the study population. A minimum of 3 consecutive SNPs had to be affected for a CNV to be called. Quality control was performed, and samples were excluded if standard deviation of LRR >0.3, B allele drift >0.01, <-0.05 waviness factor >0.05, and number of CNV calls >150. Adjacent CNVs were merged using the clean_cnv.pl with default settings.
CNVPartition

CNVPartition v3.2.0 is a plug-in software that works within GenomeStudio. CNVPartition compares observed LRR and BAF to fourteen predicted different copy number scenarios created as a bivariate Gaussian distribution \[51\]. To increase CNV calling accuracy, GC content was corrected with linear regression. For CNV calling, a minimum of 3 consecutive SNPs was required. After CNV calling, quality control metrics were plotted and samples were excluded if confidence <35, the standard deviation of LRR >0.3, and B allele deviation >0.05.

QuantiSNP

QuantiSNP 2.0 is an algorithm that incorporates the LRR and BAF in an Objective Bayes Hidden-Markov Model and uses a fixed rate of heterozygosity for every SNP \[52\]. GC files were made using the Data Integrator tool made available by University of California Santa Cruz (UCSC) Genome Bioinformatics. QuantiSNP outputs values of outlier rate, standard deviation of LRR, and standard deviation of BAF for each chromosome for every sample. Values were summed and totals were plotted to compare all individuals. Quality control cut-offs were determined to be the sum of the standard deviation of LRR >7.5, the sum of the standard deviation of BAF >3.7, and the sum of the outlier rate >0.09. All CNV calls with a Log Bayes Factor <10 were removed.

ParseCNV

ParseCNV is a software package that creates CNVRs in a dynamic case-control study design by grouping CNVs that are close in proximity and have comparable significance \[53\]. When ParseCNV is run with permutations it performs a Fisher’s Yates Shuffle to permute the array of cases and controls in place for association analysis and reports max(T) permutation test \(P\)-values that are corrected for the number of tests based on the permutation distribution. The number of permutations specified are performed on each significant CNVR detected and each observed test statistic is compared against the maximum of all permuted statistics meaning the max(T) permutation \(P\) value reflects the chance of seeing a test statistic that large, given the number of tests performed. The permutation scheme preserves the correlational structure between CNVRs and provides a less stringent correction for multiple testing where moderate values such as 0.05–0.01 can represent genome-wide significance. CanFam2.0 reference files were created to run the program with a canine data set. The quality-controlled output for each of the three calling algorithms was analyzed separately. ParseCNV was run with 10,000 permutations. A nominal significance threshold (max(T) permutation \(P\) <0.05) was used to select CNVRs for inclusion in a pathway analysis. For overlapping CNVRs detected by multiple algorithms, Benjamini-Hochberg false discovery rate correction \[54\] of the association \(P\) value was performed.

Pathway analysis and CNVR lift over

Of the CNVRs that met the nominal significance threshold (max(T) permutation \(P\) <0.05), the CNVR regions were converted from CanFam2.0 to CanFam3.1 using the UCSC lift-over tool because CanFam3.1 is the current annotated assembly of the canine genome. During the lift-over process, five CNVRs were unable to be transferred from CanFam2.0 to CanFam3.1 due to an error message regarding portions of the regions being partially deleted in the new build. One CNVR that generated an error message was able to be successfully lifted over when a new start location was assigned within 100bp of the ParseCNV generated location. The four other CNVRs start and end locations were able to be lifted over when just the start and end bp
locations were converted as points in the genome rather than trying to convert the whole region. A gene list was constructed by identifying genes that fall within the significant CNVRs as listed in the CanFam3.1 build on the UCSC and Ensembl genome browsers. A functional annotation of the gene list was processed using Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/summary.jsp) [55]. To overcome limitations in the canine genome annotation and canine annotation enrichments, genes were converted to the human ortholog and were mapped with DAVID to Homo sapiens. DAVID is an analytic biologic database that looks for meaningful clusters in large gene lists. In addition, the gene list was divided into genes found on the duplication CNVRs and genes found on the deletion CNVRs. The subset of genes associated with duplication and deletion CNVs were also analyzed separately through DAVID. DAVID pathway P-values had Benjamini correction applied.

qPCR

DNA that was available from dogs with detected CNV deletions in the homeobox pathway was analyzed compared to control samples by qPCR to assess relative copy number. For each CNVR, two control Labrador Retrievers without ACL rupture that did not have reported CNV calls overlapping the investigated CNVRs were used as control samples for qPCR. For each of the three CNVRs in the significant homeobox pathway, the individual CNV calls were mapped to detect CNV breakpoints. DNA locations used for primer design were selected to avoid repeat regions reported in CanFam3.1 and were centered in areas of maximal overlap, where all individuals with detected CNV deletions had overlapping deletions within the significant CNVR. Primers were designed using IDT primer design programs (https://www.idtdna.com/scitools/Applications/RealTimePCR/). Ethanol precipitation with sodium acetate was performed on all DNA samples previously stored in AE buffer. The qPCR was performed in triplicate in 15μl volume using SYBR Green PCR Master Mix (Applied Biosystems) in a ViiA 7 Real Time PCR system (Applied Biosystems) with 5ng of genomic DNA. Forward and reverse primer final concentrations were 300nM each. Serial dilutions were performed for each primer pair to evaluate PCR efficiency. Amplification was performed under the following conditions: one cycle at 58˚C for 2 minutes, one cycle at 95˚C for 10 minutes, 40 cycles at 95˚C for 15 seconds, and 60˚C for 1 minute. After each qPCR run, dissociation curve analysis was performed to assess qPCR specificity. Relative copy number was quantified using the $2^{-\Delta\Delta Ct}$ method [56]. A previously validated reference gene for canine studies, C7orf28B, was used to normalize against the averaged triplicate Ct values [57].

Results

Study population

Our purebred Labrador Retriever data set consisted of 164 phenotype-negative controls and 110 cases. PCA plot of 274 Labrador Retrievers showed genetic homogeneity between the case and control groups (Fig 1). Of the 274 dogs, 145 were male (90 controls, 55 cases) and 129 were female (74 controls 55 cases). The control group had a mean age of 10.5 years with a standard deviation of 1.75, while the affected group had a mean age of 6 and a standard deviation of 2.67. After genotyping, no individuals were removed due to low call rate. Quality control removed 30 dogs from the PennCNV dataset (148 controls, 96 cases), 17 from CNVPartition (154 controls, 103 cases), and 25 from QuantiSNP (144 controls, 105 cases). A total of 16 dogs were overlapping between the PennCNV and QuantiSNP excluded lists.
CNVR association analysis

Our analysis revealed no strong effect CNVRs and 46 small effect (max(T) permutation $P<0.05$) CNVRs that associated with ACL rupture. Frequencies in 42 of the associated CNVRs occurred in the population <10%, while 4 occurred at a frequency in the population 10–25%. CNVRs were identified on chromosomes 1, 2, 5, 6, 7, 9, 13, 14, 15, 18, 20, 21, 22, 23, 24, 25, 28, 30, 31, 32, 33, and 36 (Fig 2). Of these chromosomes, chromosomes 1, 6, 9, 21, and 24 had regions that were found by at least two of the calling algorithms. Regions on chromosome 6 and 24 were identified by all three algorithms. At the chromosome 6 location all three algorithms identified a duplication event that associated with cases. On chromosome 24, PennCNV and QuantiSNP called the region to be a deletion event that associated with cases, while CNVPartition identified a duplication event that associated with cases. Of the 5 CNVRs with multiple calls, all CNV associations remained significant after FDR correction.

CNVRs were widely distributed across the genome. Of the 46 CNVRs identified, 25 were deletions and 21 were duplications. All of the 46 CNVRs associated with cases. The mean size for all CNVRs was 198872bp and the median 51348bp. CNVRs ranged in size from 7926 to 1557850bp. Chromosome 1 had the largest number of CNVRs of all the chromosomes. Chromosomes that had the highest percent base pair coverage by CNVs were 6, 21, and 33 (Fig 3).

The number of significant CNVRs called by PennCNV, CNVPartition, and QuantiSNP were 20, 5, and 21, respectively (Fig 4). CNVPartition detected the longest CNVRs with an average length of 511kb, while QuantiSNP had the shortest average length at 205kb. Conversely, QuantiSNP had the highest percentage coverage of the genome with significant CNVRs covering 0.15%, while CNVPartition had the lowest coverage at 0.11%. PennCNV performed in the middle for both length and percent base pair coverage with values of 259kb and 0.13%, respectively. From the 20 dogs that were randomly selected for concordance measurements, concordance between PennCNV and QuantiSNP had a mean of 53.4% with a standard deviation of 15.3, concordance between PennCNV and CNVPartition had a mean of 7.3% with a standard deviation of 9.4, and concordance between QuantiSNP and CNVPartition had a mean of 51.4% with a standard deviation of 15.
Many annotated genes were found within these CNVRs. None of the associated CNVRs overlapped with significant ACL rupture risk loci discovered previously in the same Labrador Retriever population GWAS [14]. In 46 CNVRs, a total of 152 genes were identified (Table 1). The chromosome 6 CNVR that was identified by all three algorithms contained the genes RNPC3 and AMY2B. The chromosome 24 CNVR identified by all three algorithms contained the genes OSBPL2, ADRM1, and RPS21. Collagen genes COL9A3 and COL6A1 were within CNVRs on chromosomes 24 and 31, respectively. There were 12 CNVRs without any known genes in the CanFam3.1 assembly.

Pathway analysis

DAVID grouped 19 genes into one homeobox domain significant biologic cluster. The Benjamini corrected P-value for the significant cluster was 6.6E-13. The 19 genes were NKK6-2, EVX1, EVX2, HoxA3, HoxA4, HoxA5, HoxA6, HoxA7, HoxA9, HoxA10, HoxA11, HoxA12, HoxA13, HoxD3, HoxD4, HoxD8, HoxD9, HoxD11, and HoxD12. These 19 genes were found in 3 CNVRs located on chromosomes 14, 28, and 36. HoxA, HoxD, and NKK6-2 deletion CNVs were all identified by only one calling algorithm. PennCNV detected the HoxA and HoxD deletion CNVs while QuantiSNP detected the NKK6-2 deletion CNV. The subset of
genes found in duplication CNVRs grouped into a pathway of cell cycle, cell division, and mitosis regulators that was not significant. The subset of genes found in deletion CNVRs clustered the same 19 genes in a grouping with a lower Benjamini corrected P-value of 8.7E-16.

Homeobox structural variation and knee morphology

PTS and rTTW values were measured from case and control radiographs. PTS and rTTW are quantitative parameters that describe morphology of the proximal tibia and have been shown to influence knee biomechanics by increasing load on the ACL, thus promoting ligament degeneration and fiber rupture [45, 58]. Radiographs were obtained for all dogs, but only a subpopulation of radiographs were determined to be of sufficient quality for measurement of PTS and rTTW, which included 125 controls (76%) and 57 cases (62%). Median PTS and rTTW were calculated for case and control groups and all dogs with any HoxA, HoxD, or NKX6-2 CNVR deletion were excluded. The median PTS and range for the control and case
groups was 28.0 (20.9–37.6) and 29.0 (20.9–35.0), respectively (Table 2). The median rTTW and range for the control and case groups was 0.70 (0.53–0.96) and 0.65 (0.45–1.00), respectively (Table 2). There was no significant difference between case and control group TPA. rTTW was significantly different between case and control groups ($P = 3.0E-4$).

Of the dogs with radiographs that passed quality control, we found 7 (5 controls, 2 cases) dogs had $HoxA$ CNVR deletions, 3 (1 control, 2 cases) dogs had $HoxD$ CNVR deletions, 3 case dogs had both $HoxA$ and $HoxD$ CNVR deletions, and 1 case had CNVR deletions in $HoxA$, $HoxD$, and $NKX6-2$. PTS values in dogs with different types of homeobox CNVR deletions varied. PTS median and range was 25.0 (22.0–28.1) for dogs with $HoxA$ deletions, 28.1 (28.0–28.1) for dogs with $HoxD$ deletions, and 29.3 (25.0–31.7) for dogs with $HoxA$ and $HoxD$ deletions. Dogs with $HoxA$ deletions had a median rTTW greater than the control group. Dogs with $HoxD$ and $NKX6-2$ CNVR deletions had lower rTTW values, similar to the case group.

### qPCR analysis of homeobox pathway CNVRs

Of dogs with homeobox CNV deletions, additional DNA for qPCR analysis was available for 3 cases with $HoxA$ (2 had radiographic measurements), 3 cases with $HoxD$ (1 had radiographic measurements), and 2 cases with $NKX6-2$ (none had radiographic measurements) CNVs. Quantitative PCR (qPCR) was performed to study the three CNVRs containing genes found in the significant homeobox pathway. For each of the three CNVRs, individual CNV calls were mapped to identify shared breakpoints. The chromosome 14 ($HoxA$) CNVR and chromosome 28 ($NKX6-2$) CNVR did not have distinct shared CNV breakpoints amongst individuals with detected CNV deletions. A method of relative quantification was used to estimate copy number at each location. Expected outcomes for a gene occurring in normal copy number of two would equal 1. A gene with a single copy loss would yield a relative copy change of 0.5. If a...
Table 1. Non-contact ACL rupture associated CNVRs identified in the Labrador Retriever using three CNV calling algorithms.

| Chromosome | CNVR Start | CNVR End | Length (bp) | Max(T) Permutation P-value for Deletions that Associate with Cases | Max(T) Permutation P-value for Duplications that Associate with Cases | False Discovery Rate Corrected P-value for Overlapping CNVRs | Number of Cases with Deletion / Number of Diploid Cases | Number of Controls with Deletion / Number of Diploid Controls | Number of Cases with Duplication / Number of Diploid Cases | Number of Controls with Duplication / Number of Diploid Controls | Algorithm that Detected the CNVR | Genes | Papers Reporting Overlapping CNVRs |
|------------|------------|----------|-------------|------------------------------------------------------------------|------------------------------------------------------------------|-----------------------------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------|----------------------------------|
| 1          | 10,203,781 | 10,214,038 | 10,258      | 0.0254                                                          |                                                                  |                                                                 | 4/78                                                                 | 0/123                                                                 | 0/78                                                                 | 0/123                                                                 | CNVPartition   |                                                |
| 1          | 11,323,575 | 11,368,842 | 45,268      | 0.0242                                                          |                                                                  |                                                                 | 1/87                                                                 | 2/143                                                                 | 8/87                                                                 | 3/143                                                                 | PennCNV        |                                                |
| 1          | 14,563,023 | 14,575,286 | 12,264      | 0.0233                                                          |                                                                  |                                                                 | 4/92                                                                 | 0/148                                                                 | 0/92                                                                 | 0/148                                                                 | PennCNV        | KIAA1468                                      |
| 1'         | 16,281,556 | 16,337,721 | 56,166      | 0.0133                                                          | 0.0133                                                          |                                                                  | 0/100                                                                 | 0/144                                                                 | 5/100                                                                 | 0/144                                                                 | QuantiSNP      |                                                |
| 1'         | 16,320,005 | 16,337,721 | 17,717      | 0.0080                                                          | 0.0133                                                          |                                                                  | 0/91                                                                 | 0/148                                                                 | 5/91                                                                 | 0/148                                                                 | PennCNV        |                                                |
| 1          | 121,419,250| 121,446,894| 27,645      | 0.0221                                                          |                                                                  |                                                                 | 1/98                                                                 | 2/141                                                                 | 6/98                                                                 | 1/141                                                                 | QuantiSNP      |                                                |
| 2          | 11,600,518 | 11,635,886 | 35,369      | 0.0270                                                          |                                                                  |                                                                 | 10/84                                                                 | 5/137                                                                 | 2/84                                                                 | 6/137                                                                 | PennCNV        | SKD1A1                                        |
| 2          | 85,122,962 | 85,238,711 | 115,750     | 0.0493                                                          |                                                                  |                                                                 | 12/73                                                                 | 8/121                                                                 | 11/73                                                                 | 19/121                                                                 | PennCNV        | CASZ1, APITD1, PEX14                         |
| 5          | 32,207,352 | 32,374,761 | 167,410     | 0.0292                                                          |                                                                  |                                                                 | 4/101                                                                 | 0/144                                                                 | 0/101                                                                 | 0/144                                                                 | QuantiSNP      | CHRN1, GABARAP, CTDNEP1, CLDN7, ELPS, YBX2, EIF5A, NEURL4, ACAPI, TNK1, PLLCR1, NLGN2, SLC2A4, ACADVL, GPS2, SPEM1, SPEM2, TMEM102, FGF11 |
| 5          | 60,282,278 | 60,342,624 | 60,347      | 0.0319                                                          |                                                                  |                                                                 | 4/101                                                                 | 0/144                                                                 | 0/101                                                                 | 0/144                                                                 | QuantiSNP      | ESPN, PLEKHG5, TNFRSF25                       |
| 6          | 38,432,771 | 39,001,830 | 569,060     | 0.0013                                                          |                                                                  |                                                                 | 1/88                                                                 | 1/147                                                                 | 7/88                                                                 | 0/147                                                                 | PennCNV        | PDPK1, NTNS3, ABCA3, E4F1, TRAF7, PKD1, GZF598, RARMB, ECII, GFER, PGP, NOXO1, CCNF, TBL3, RNPS1, TSC2, CASKIN1, NDUFB10, DNAS11, MLST8, RPL35, NT5H1, SEPX1, SLC9A3, ATP6V0C, TEDC2, RNPS1, BRCDC9, MSRBI |

(Continued)
| Chromosome | CNVR Start | CNVR End | Length (bp) | Max(T) Permutation P-value for Deletions that Associate with Cases | Max(T) Permutation P-value for Duplications that Associate with Cases | False Discovery Rate Corrected P-value for Overlapping CNVRs | Number of Cases with Deletion / Number of Diploid Cases | Number of Controls with Deletion / Number of Diploid Controls | Number of Cases with Duplications / Number of Diploid Cases | Number of Controls with Duplication / Number of Diploid Controls | Algorithm that Detected the CNVR | Genes Reporting Overlapping CNVRs |
|------------|------------|----------|-------------|---------------------------------------------------------------|---------------------------------------------------------------|----------------------------------------------------------|-----------------------------------------------|-----------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------|-----------------------------|
| 6’         | 45,281,123 | 46,342,370 | 1,061,248   | 0.0043                                                      | 0.0081                                                      | 0/71                                                     | 2/118                                             | 34/71                                   | 24/118                                             | QuantiSNP                        | Chen et al. 2009, Nicholas et al. 2009, Nicholas et al. 2011, Berglund et al. 2012, Molin et al. 2014 |
| 6’         | 45,595,573 | 47,042,969 | 1,447,397   | 0.0054                                                      | 0.0081                                                      | 0/68                                                     | 0/127                                             | 28/68                                   | 21/127                                             | PennCNV                          | AMY2B, RNPC3                      |
| 6’         | 46,949,172 | 47,027,777 | 78,606      | 0.0483                                                      | 0.0483                                                      | 0/59                                                     | 0/104                                             | 36/59                                   | 37/104                                             | CNVPartition                      | AMY2B, RNPC3                      |
| 7          | 535,999    | 675,949   | 139,971     | 0.0322                                                      |                                                             | 0/101                                                   | 1/143                                             | 4/101                                   | 0/143                                             | QuantiSNP                        | PPP1R12B                         |
| 7          | 2,864,598  | 2,917,287 | 52,690      | 0.0023                                                      |                                                             | 9/87                                                    | 1/147                                             | 0/87                                    | 0/147                                             | PennCNV                          | TMEM105, SLC38A10, ENTHD2, AZI1, AATK, BAIAP2, CEP131, NDUFAF8, TEP5IN, CHMP6 |
| 9’         | 732,580    | 997,748   | 265,168     | 0.0464                                                      | 0.0464                                                      | 6/89                                                    | 2/144                                             | 1/89                                    | 2/144                                             | PennCNV                          | TMEM105, SLC38A10, ENTHD2, AZI1, AATK, BAIAP2, CEP131, NDUFAF8, TEP5IN |
| 9’         | 747,275    | 980,264   | 232,990     | 0.0401                                                      | 0.0464                                                      | 5/85                                                    | 1/122                                             | 2/85                                    | 1/122                                             | CNVPartition                      | TMEM105, SLC38A10, ENTHD2, AZI1, AATK, BAIAP2, CEP131, NDUFAF8, TEP5IN |

(Continued)
| Chromosome | CNVR Start (bp) | CNVR End (bp) | Length (bp) | Max(T) | Permutation P-value for Deletions that Associate with Cases | Number of Cases with Deletion / Number of Diploid Cases | Algorithm that Detected the CNVR | Genes | Papers Reporting Overlapping CNVRs |
|------------|----------------|--------------|------------|--------|------------------------------------------------------------|------------------------------------------------------|--------------------------------|-------|----------------------------------|
| 9          | 36,302,228     | 36,444,206   | 141,979    | 0.0161 | 1/84                                                       | 11/84                                               | PennCNV                         | CA4, PSMG4, ZNF141, GFA, P2IR | Nicholas et al. 2009, Nicholas et al. 2011, Berglund et al. 2012, Molin et al. 2014 |
| 13         | 21,954,922     | 22,930,414   | 97,492     | 0.0136 | 0.0426                                                     | 21/88                                               | QuantiSNP                       | PTP4A3                         | Chen et al. 2009, Nicholas et al. 2009, Nicholas et al. 2011, Berglund et al. 2012, Molin et al. 2014 |
| 14         | 40,729,369     | 40,826,145   | 10,766     | 0.0257 | 3/79                                                       | 7/79                                                | PennCNV                         | OR2T2                          | Chen et al. 2009, Nicholas et al. 2011, Berglund et al. 2012, Molin et al. 2014 |
| 18         | 42,199,134     | 42,331,627   | 142,494    | 0.0152 | 0.049                                                      | 1/39                                                | PennCNV                         | ETV1, HOXA3, HOXA5, HOXA7, HOXA10, HOXAI1, CEBPA, CSMD2 | Chen et al. 2009, Nicholas et al. 2011, Berglund et al. 2012, Molin et al. 2014 |
| 20         | 45,012,802     | 45,030,642   | 17,841     | 0.0050 | 0.026                                                      | 0/99                                                | QuantiSNP                       | SERPINE1, STAG1, SCG5C2, ADAM12, ADAM12, RNA1 | Nicholas et al. 2011, Berglund et al. 2012, Molin et al. 2014 |
| 21         | 52,280         | 59,427       | 7,147      | 0.0396 | 0.0345                                                     | 0/52                                                | PennCNV                         | OSBPL2, ADRB1, RPS11             | Chen et al. 2009, Nicholas et al. 2011, Berglund et al. 2012, Molin et al. 2014 |

(Continued)
| Chromosome | CNVR Start | CNVR End | Length (bp) | Max(T) Permutation P-value for Deletions that Associate with Cases | Max(T) Permutation P-value for Duplications that Associate with Cases | False Discovery Rate Corrected P-value for Overlapping CNVRs | Number of Cases with Deletion / Number of Diploid Cases | Number of Cases with Duplications / Number of Diploid Cases | Number of Controls with Deletion / Number of Diploid Controls | Number of Controls with Duplications / Number of Diploid Controls | Algorithm that Detected the CNVR | Genes | Papers Reporting Overlapping CNVRs |
|------------|------------|----------|-------------|---------------------------------------------------|-------------------------------------------------|-------------------------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|--------|----------------------------------|
| 24'        | 46,306,925 | 46,314,851 | 7,927       | 0.0126                                           | 0.0045                                          | 0.0045                                          | 5/100                           | 0/142                           | 0/100                           | 2/142                           | QuantiSNP                       | OSBPL2, ADRM1, RPS21            | Chen et al. 2009, Nicholas et al. 2011 |
| 24         | 47,484,099 | 47,557,195 | 73,097      | 0.0054                                           | 1/98                                           | 2/142                                           | 6/98                            | 0/142                           | 0/142                           |                                | QuantiSNP                       | PRPP6, SOX18, TCEA2, RG519, OPL1 | |
| 25         | 17,983,587 | 18,005,251 | 21,665      | 0.0240                                           | 6/99                                           | 1/143                                           | 0/99                            | 0/143                           | 0/143                           |                                | QuantiSNP                       | GJA3                             | |
| 28         | 40,514,129 | 40,715,077 | 200,949     | 0.0493                                           | 5/99                                           | 1/139                                           | 1/99                            | 4/139                           |                                |                                | QuantiSNP                       | GPR123, ZNF511, NKX6-2, TTDG0, INPP5A, CFAF46 | Nicholas et al. 2011 |
| 30         | 23,898,138 | 23,923,042 | 25,905      | 0.0139                                           | 0/100                                         | 0/144                                           | 5/100                           | 0/144                           | 0/144                           |                                | QuantiSNP                       | SLC7M                            | |
| 31         | 39,364,930 | 39,428,602 | 65,673      | 0.0332                                           | 4/97                                          | 0/140                                           | 4/97                            | 4/140                           | 4/140                           |                                | QuantiSNP                       | PCBP3, PTCD, COX6A1             | |
| 32         | 28,274,625 | 28,487,185 | 212,561     | 0.0291                                           | 4/101                                         | 0/144                                           | 0/101                           | 0/144                           | 0/144                           |                                | QuantiSNP                       | SGM52, CYP2U1, HADH             | |
| 33         | 551,701    | 580,231   | 28,531      | 0.0369                                           | 5/87                                         | 1/143                                           | 4/87                            | 4/143                           |                                |                                | PennCNV                         |                                 | |
| 33         | 607,740    | 1,017,726 | 400,986     | 0.0331                                           | 4/99                                         | 0/138                                           | 2/99                            | 6/138                           |                                |                                | QuantISNP                       | EPHA3, PROS1                    | |
| 36         | 2,795,707  | 2,804,494 | 8,888       | 0.0353                                           | 5/91                                         | 1/147                                           | 0/91                            | 0/147                           |                                |                                | PennCNV                         |                                 | |
| 36         | 16,189,091 | 16,198,074 | 8,984       | 0.0213                                           | 6/86                                         | 7/141                                           | 4/86                            | 0/141                           |                                |                                | PennCNV                         | SLC25A12                        | |
| 36         | 19,876,345 | 19,978,344 | 102,000     | 0.0020                                           | 9/87                                         | 1/147                                           | 0/87                            | 0/147                           |                                |                                | PennCNV                         | EVX2, HOXD1, HOXD4, HOXD8, HOXD9, HOXD10, HOXD11, HOXD12 | |

**Note:** Start and end CNVR positions and genes located within those regions are reported on the CanFam3.1 canine assembly. PennCNV [50], CNVPartition [51], and QuantiSNP [52], were used for CNV calling. Association analysis was performed using ParseCNV [53]. Max(T) permutation test P-values were calculated by ParseCNV using 10,000 permutations and were corrected for the number of tests based on the permutation distribution.

*Overlapping CNVRs confirmed by multiple algorithms. Benjamini-Hochberg false discovery rate correction [54] of P-values is also reported for overlapping CNVRs detected by multiple algorithms.

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gene has two copies deleted, then the expected relative copy number would be 0. When compared to two control samples (2 Labrador Retrievers with no ACL ruptures and no reported CNV aberration in the region), the three dogs with the HoxA CNVR deletion had relative copy numbers of 0.55 ± 0.009, 0.80 ± 0.008, and 0.85 ± 0.014 (Fig 5). The dog with a relative copy number of 0.55 ± 0.009 for the HoxA CNVR deletion had a PTS of 25.0 and rTTW of 0.56 while the dog with a relative copy number of 0.80 ± 0.008 in the HoxA CNVR deletion had a PTS of 22.0 and rTTW of 0.85. The dogs with NKX6-2 CNVR deletions had qPCR relative copy numbers of 0.55 ± 0.003 and 0.75 ± 0.019. The third CNVR in the homeobox pathway containing the HoxD gene cluster had 3 affected dogs with reported deletions from the CNV calling that had qPCR relative copy numbers of 0.37 ± 0.002, 0.92 ± 0.012, and 0.95 ± 0.013. Deletions were not validated with qPCR in 2 of the 3 dogs with HoxD CNVs called from SNP array data. No radiographic measurements were available on these 2 dogs. The affected dog with a validated HoxD decreased relative copy number (0.37 ± 0.002) had a PTS of 29.3 and rTTW of 0.63.

**Discussion**

The aim of this study was to identify candidate CNVs that contribute to the genetic risk of ACL rupture in dogs. By using one breed of dog, creation of a custom cluster file, and use of three different CNV calling algorithms, no strong effect CNVRs were identified. However, we found 46 small effect ACL rupture associated CNVRs, the majority occurring with <10% frequency in the population. Although the majority of CNVs have low frequency within this population of Labrador Retrievers they may represent independent de novo CNVs events. De novo CNVs are non-inherited sporadic structural mutations meaning CNVs that are present in offspring but are not present in their parents [33]. This form of non-inherited structural mutation

| Dog Groups | PTS (degrees) | rTTW | Number of Dogs with Radiographic Measurements | Number of Dogs with Radiographic Measurements and Additional DNA for qPCR Validation |
|------------|---------------|------|---------------------------------------------|---------------------------------------------------------------------------------|
| Controls¹ | 28.0 (20.9–37.6) | 0.70 (0.53–0.96) | 114 | Controls Cases Cases |
| Cases² | 29.0 (20.9–35.0) | 0.65 (0.45–1.00) | 56 | |
| HoxA CNVR deletion | 25.0 (22.0–28.1) | 0.75 (0.56–1.00) | 5 | 2 | 2 |
| HoxD CNVR deletion | 28.1 (28.0–28.1) | 0.64 (0.63–0.83) | 1 | 2 | |
| HoxA and HoxD CNVR deletions | 29.3 (25.0–31.7) | 0.63 (0.62–0.71) | 3 | 1 | |
| HoxA and HoxD and NKX6-2 CNVR deletions | 26.0 | 0.62 | 1 | |

**Note:** PTS and rTTW were measured from lateral knee radiographs [44–46]. Data represent median and (minimum–maximum).

¹Control and case groups calculated PTS and rTTW excluded all dogs with any HoxA, HoxD, or NKX6-2 CNVR deletion.

²rTTW was significantly different between cases and controls (P = 3.0E-4).

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occurring in a small number of affected individuals (<10) at the same genomic location or affecting similar biologic pathways could be very significant because the rate of sporadic structural mutation is much lower than the rate of CNV inheritance. Therefore, CNVs present in only a handful of cases can potentially imply subtle but strong disease association [33, 59]. In humans, there are several examples where low frequency de novo CNVs have been found to significantly contribute to complex polygenic disease risk [60–63].

A list of 152 annotated genes from the associated CNVRs formed a significant cluster of 19 genes into a biologic pathway that regulates skeletal morphology. These 19 genes were located in 3 CNVRs identified by only one calling algorithm. PennCNV detected two of these CNVRs and QuantiSNP detected the other CNVR. This clustering was enhanced when genes associated with only CNV deletions were considered. This enriched pathway included homeobox genes of the Hox classes A and D. Hox genes encode transcription factors that are essential embryonic regulators of body patterning and limb formation. The vertebrate genome has 39 Hox genes that are separated into four distinct chromosomal clusters, HoxA-HoxD [64, 65]. Hox expression during embryogenesis is complex and precise timing, quantity, and spatial distribution is crucial for proper development [66]. Several human and mouse limb abnormalities have been linked to mutations and CNVs that alter Hox genes or their regulatory elements [67]. Specifically, HoxA and HoxD have been reported to be the most important clusters for proper limb development [68]. Conditions such as synpolydactyly and split hand/foot malformation have been shown to be a consequence of HoxD mutations [69].

Association of canine PTS and ACL rupture is controversial; some studies have linked a greater PTS with higher incidence of ACL rupture [45, 70]. In the dogs of the present study there was no significant difference between case (n = 56) and control (n = 114) group PTS. Smaller relative tibial tuberosity widths have been associated with ACL rupture in dogs [45], likely a consequence of increased anterior tibial thrust under load. In this study, affected dogs...
had significantly smaller rTTW than controls. The HoxD (n = 3), HoxA and HoxD (n = 3), and HoxA, HoxD and NKX6-2 (n = 1) deletion groups had particularly low rTTW values. Our data suggest these CNVR deletions may play a role influencing tibial tuberosity width in a subset of ACL rupture dogs. However, these results should be interpreted with caution because of small sample size and wide range of measured values. Investigation of additional dogs to increase power and permit statistical analysis is needed to further understand these observations and confirm significance of these associations.

Tibial and femoral conformation is a risk factor for canine ACL rupture. Effects on knee joint incongruity, changes in articular contact areas, altered joint angles, or increased stress loading on the ACL may all be important [71, 72]. In human beings, lateral femoral condyle height, as well as anterior-posterior and medial-lateral tibial plateau distances, have been shown to significantly differ between people with and without ACL rupture [73]. Although Hox genes primarily control patterning and growth of skeletal elements, several studies have demonstrated Hox genes involvement in cartilage differentiation and synovial joint organization [74, 75]. Hox mutations can disrupt collagen fibril formation and extracellular matrix production that results in abnormal chondrogenesis by affecting downstream Shh and Sox9 expression [75, 76]. Mechanisms that affect passive knee joint stabilizers (menisci, joint capsule, collateral ligaments), changes to ACL fibroblasts, collagen fibril abnormalities, or the synovial sheath have also been suggested to influence canine ACL rupture [71]. With Hox developmental pathways and their downstream effects being so widespread it is not possible to determine a specific mechanism associated with these CNV deletions that increase risk for ACL rupture without further research.

With only 18 affected dogs possessing homeobox CNVR deletions, reduced signaling of this pathway is not the only genetic mechanism influencing risk of canine ACL rupture. ACL rupture is a complex polygenic disease [14, 21, 28, 77]. Our results suggest alterations in HoxA, HoxD and NKX6-2 expression may influence risk of ACL rupture in a proportion of dogs. Further investigation of bone and joint morphology is needed to robustly define and validate the relationship between Hox deletions and ACL rupture.

CNVs associated with ACL rupture likely influence multiple physiological pathways, as genes from the significant homeobox pathway represent only 3 of the 46 ACL rupture associated CNVRs. Areas of substantial interest for future investigation would include CNVRs with the largest base pair coverage per chromosome, CNVRs that are verified by multiple programs, and CNVRs containing genes associated with collagen matrix. The 2 CNVRs that had overlapping areas and confirmed by all 3 algorithms were located on chromosomes 6 and 24. Overlapping CNVRs have been reported in previous canine CNV discovery papers in these same regions supporting the observation that these genomic regions exhibit structural variation (Table 1). Of the overlapping regions on chromosome 6, 2 CNVRs contained the genes AMY2B and RNPC3. AMY2B encodes for pancreatic amylase. In the dog, AMY2B has historically been associated with increased copy number and domestication [78–80]. Domestic dogs have various increases in copy number of AMY2B when compared to wolves [79]. AMY2B duplications result in high amylase activity which increases starch digestion efficiency. More recent canine domestication research has identified a large duplication in the genomic region distal and separate from AMY2B that is centered on RNPC3 [81]. RNPC3 encodes for a small nuclear ribonucleoprotein (snRNP) that is a component of the minor U12-dependent spliceosome [82]. Spliceosomes regulate pre-mRNA processing and removal of non-coding sequences and introns. The U12-dependent spliceosome removes U12-type introns that represent less than 0.5% of all introns. Although U12-type introns are found at much lower frequencies, U12 type splicing is still essential for gene expression, but much of its importance is not yet characterized. Alterations to the U12-dependent spliceosome result in abnormal growth,
developmental defects, and disrupted gene expression [83]. RNPC3 has been linked to dwarfism in humans through defective pituitary somatotroph development and subsequent growth hormone deficiency [84]. It is unclear by what biologic mechanism a duplication CNV in RNPC3 might influence risk for ACL rupture, although alterations to copy number of RNPC3 in dogs potentially could result in global phenotypic effects on body size as suggested by its association to the canine domestication syndrome [81]. Two of the three overlapping regions were roughly 1Mb in length harboring only the AMY2B and RNPC3 genes. It is also possible that this region on chromosome 6 is functioning as a regulatory region influencing expression of genes occurring at other chromosomal locations.

The CNVR on chromosome 24 was called by all three algorithms. OSBPL2 appeared in all 3 regions and COL9A3 was only found in one of those regions. OSBPL2 is a lipid binding protein that is an important regulator of intracellular cholesterol homeostasis through lipid metabolism and transport. Knockout OSBPL2 bama miniature pigs have hypercholesterolemia, increase in adipocytes, with obesity phenotypes [85]. The link between decreased OSBPL2 expression and obesity in an animal model is interesting given obesity is a known epidemiological risk factor for canine ACL rupture and two of the chromosome 24 CNVRs were deletions. COL9A3 encodes for the α3 chain of type IX collagen. In humans, mutations in COL9A3 have been associated with two genetically and phenotypically heterogeneous types of osteochondrodysplasia, multiple epiphyseal dysplasia (MED) and pseudoachondroplasia (PSACH) [86–88]. MED is clinically characterized by mild to moderate short stature, abnormally small and/or irregular epiphyses, and early-onset OA predominantly in the hip and knees. PSACH is characterized by disproportionate short-limbed short stature, joint laxity, long bone deformities and early-onset OA. Both MED and PSACH can have varying levels of phenotypic severity. Interestingly, COL9A3 mutations have been linked to oculoskeletal dysplasia (OSD) in the Labrador Retriever [89]. OSD dogs can have a range of phenotypes which include short-limbed dwarfism, delayed epiphyses development, cataracts, retinal dysplasia, and retinal detachment [90]. One study performed candidate gene analysis for ACL rupture in the Newfoundland including COL9A3 as a candidate gene because of its known associations with primary arthritis and osteochondrodysplasias in humans [91]. No significant association was found with COL9A3 and ACL rupture in the population of 90 Newfoundland dogs. Another candidate gene study found no association with COL9A3 and ACL rupture in 12 Boxers [92]. Despite the Newfoundland and Boxer representing high-risk ACL rupture breeds, the candidate gene approach is most successful at identifying large effect mutations associated with monogenic traits and is limited at complex disease small-effect variant discovery [93].

Although the CNVR on chromosome 31 containing COL6A1 was only called by one program, COL6A1 has interesting associations with tendon and synovial joint biomechanics and OA. The COL6A1 gene encodes for the α1 chain of type VI collagen. Collagen VI is a non-fibrillar collagen found in extracellular matrix of muscle and most connective tissues. Mutations in COL6A1 are most famously linked to a wide range of muscular dystrophies of varying severity in humans [94]. These collagen type VI disorders can have tendon involvement [94]. Normal tendon is typically composed of a few fibroblasts in a dense extracellular matrix of type I collagen fibrils with surrounding scattered collagen VI. In humans, severe myopathy caused by a collagen VI mutation is called Ullrich congenital muscular dystrophy (UCMD). Tendons of UCMD patients can have changes to pericellular matrix organization causing an abnormal accumulation of collagen type VI and altered distribution of collagen type I which results in dysfunctional fibrillogenesis [95]. Subsequently, severely affected UCMD humans can have tendon fibrils with irregular profiles and decreased mean diameter. COL6A1 knockout mice have similar tendon changes with disorganized collagen fibers with abnormal fibril structure and decreased fibril diameter [94, 96]. These mice have a ~2.5 increase in tendon fibril
density which results in biomechanical changes with significant reduction in maximum load and stiffness [96]. *COL6A1* knockout mice also have significant changes to their trabecular bone structure with decreased bone volumes that have been noted in the proximal tibia [97]. These knockout mice also experience accelerated OA development due to loss of stiffness in the articular cartilage pericellular matrix revealing type VI collagen has a role in regulating the physiology of chondrocytes and physiology of the synovial joint [98]. A candidate gene study included *COL6A1* SNPs to investigate the genetics of ACL rupture in a population of 271 Newfoundlands, 289 Labrador Retrievers, 138 Rottweilers, and 51 Staffordshire Bull Terriers [21]. This candidate gene study found no significant associations between *COL6A1* and ACL rupture. However, advanced bioinformatic analyses in humans continues to find significant associations with OA and *COL6A1* in differentially expressed genes and pathways [99, 100].

This study found that CNVRs associate with cases, suggesting CNVs are not acting in a protective manner in control dogs, but rather are disrupting gene dosage or gene regulation to increase risk of ACL rupture. In theory, CNVs could similarly be found to associate with control status and offer adaptive advantages to decreased disease risk. However, the extensive amount of CNV disease association studies in humans and dogs supports the idea that non-adaptive or disadvantageous CNVs much more frequently associate with disease by either having subtle effects of disease predisposition or directly causing disease compared to the very few reports of beneficial control associated CNVs [31, 101, 102]. Therefore, having all identified CNVRs associate with case status is not unusual. Multiple other genes found in the CNVRs associated with ACL rupture influence cell cycle regulation, cellular signaling, apoptosis pathways, and translation/transcription regulation. Another interesting feature of the 46 associated CNVRs is that 12 of them do not contain genes that are annotated on the CanFam3.1 reference assembly. This may be due to poor annotation of the current canine genome assembly. Alternatively, these regions could be acting in a regulatory fashion to alter gene expression.

We determined that the standard cluster file provided by Illumina did not fit our Labrador Retriever population very well. The training sample set used to generate the commercially available cluster file represented over 20 diverse dog breeds. Creating a user-generated custom cluster file is recommended when samples are from a unique or isolated population [48, 49]. When CNV calling is performed from a SNP array, it is critical the cluster file accurately represents the data set, because the cluster positions are used to compute both LRR and BAF [49]. LRR and BAF are the essential input values which PennCNV [50], CNVPartition [51], and QuantiSNP [52] use to detect CNVs. Control samples were the only samples used to create the cluster file because cases may have a large amount of CNVs for biological reasons and those should not be included when defining cluster centroid positions [48]. Creating a breed-specific cluster file from the control samples with high call rate increased detection and accuracy of CNV calling.

Three algorithms were selected to process the same raw data set to produce a more comprehensive CNV analysis because of the known differences in CNV calling results produced by each analytic tool [103]. To our knowledge, there are no previous publications comparing and reporting performance of these three CNV algorithms in the dog. Given the underlying polygenic architecture of ACL rupture in the Labrador Retriever, we aimed to detect as many small subtle effect genetic contributions to disease risk without missing associations due to use of too few CNV calling algorithms. PennCNV, CNVPartition, and QuantiSNP were selected because together they offered an extensive CNV detection procedure although use of multiple algorithms may increase the likelihood of detecting false positives. Each program generated quality control variables that allowed for the exclusion of noisy samples. Published concordance rates of CNV calls from the same sample generated from any combination of two algorithms is 25–50% [104]. In this study, PennCNV and QuantiSNP (53.4%±15.3) had
comparable concordance to QuantiSNP and CNVPartition (51.4±15). However, PennCNV and CNVPartition had very low concordance (7.3±9.4). Calling programs vary in the underlying mathematical models. An algorithm’s performance is influenced by the noise specific to that experiment, with factors such as the lab processing the sample, the array the samples are processed on, and the quality of the individual sample impacting results. Algorithm performance was evaluated by comparing the average CNVR length detected and comparing the overall base pair percentage coverage of the entire canine genome. With our data, CNVPartition called the least amount of CNVs, but those that were called were on average larger than the other algorithms. QuantiSNP called the most CNVs that were on average the shortest. Studies in humans and horses have reported PennCNV and QuantiSNP to have good concordance and similar amounts of CNVs calls, while CNVPartition has been reported to detect a small number of CNVs and have low concordance with PennCNV and QuantiSNP [105, 106]. This study suggests these algorithms perform similar in the dog as previously reported in other species.

Initially PennCNV was run without a GC correction. Many of the samples did not meet a waviness factor cut off of < -0.1 and > 0.1. PennCNV and other programs have the ability to fit regression models with GC content to correct for variation in hybrid intensity that is related to the probe’s location in the genome. Genomic waves are not platform-specific and can prevent accurate CNV detection [107]. The waviness factor accounts for all of the signal fluctuation of a genotyped sample throughout the entire genome. The GC waviness factor measures the fraction of fluctuation that is explained by local GC content of the DNA. Including the GC content of the canine genome during calling greatly reduced the measured waviness factor for every sample and proved to be essential for accurate CNV calling of our data. GC correction was applied during CNV calling using PennCNV, CNVPartition, and QuantiSNP.

CNVRs containing genes found in the significant homeobox signaling pathway were evaluated with relative qPCR. Only a subset of the individuals with detected CNV deletions were able to be assessed because of limited sample DNA. Ambiguity exists in the interpretation of the qPCR results. The affected individuals did not produce expected relative copy numbers of 0 and 0.5 when homozygous and heterozygous deletions are present, respectively. Relative copy number estimated by qPCR in individuals with reported CNV called deletions ranged from 0.37–0.95. Canine CNV studies have reported two different categories of CNVRs. Canine CNVRs can be categorized as simple or complex based on the structural complexity of the CNVs comprising that region. Simple CNVRs have consistent patterns of breakpoints across individuals. Complex CNVRs have substantial variation in breakpoints between individuals along with spatial heterogeneity in copy number [38]. Within a particular complex CNVR, interesting patterns of alternating gains and losses have been observed. CNVs in dogs occurring in areas of segmental duplications are more likely to be complex [36, 108]. Segmental duplications are regions at least 90% identical at two or more loci that serve as hotspots of genomic rearrangement and CNV formation [38, 108]. One shortcoming of CNV calling using the CanineHD array is a decreased ability to identify short duplications/deletions due to the 13kb probe spacing [37]. Therefore, if there is a large complex CNV that is comprised of smaller regions of alternating copy number state occurring consecutively, CNV calling and genotyping on an array with 13kb probe spacing may not correctly represent the complexity of that region [36]. Future investigation of these regions should include short-read whole genome sequencing (WGS) that provides higher resolution of CNV detection and breakpoint assessment. Because the chromosome 14 (HoxA) CNVR and chromosome 28 (NKX6-2) CNV did not have shared CNV breakpoints amongst individuals, it provides evidence they are architecturally complex CNVRs. Having substantial variation between individual breakpoints or spatial heterogeneity in copy number would make qPCR validation challenging without precisely
defining the fine-scale architectural complexity occurring at that location. A previous qPCR analysis of two CNVRs that were located in segmental duplications resulted in relative copy number changes that were not the expected 2 for a gain or 0.5 for a loss. Rather, smaller incremental relative changes were observed between individuals [38]. For example, if an individual with a loss when compared to a reference sample containing 3 copies would yield a relative copy number of 0.66. If the average copy number state for our Labrador Retriever population at these locations is greater than two copies, the qPCR results could be validating true relative signal reductions in the affected individuals. A previous canine CNV discovery paper reported a CNVR in the chromosome 28 (NKX6-2) CNVR locus providing support of a CNV present at this location [36]. None of the CNVRs found in the present study overlapped with previously reported canine ACL rupture risk loci. All detected CNVs were only associated at max(T) permutation test $P<0.05$, which suggests that CNV contribution to ACL rupture risk in the Labrador Retriever would not meet genome-wide significant thresholds during GWAS.

Major limitations with the supporting evidence regarding the ACL rupture associated homeobox developmental pathway are that the contributing genes are within CNVRs that were only detected by single calling algorithms and that the chromosome 36 (HoxD) and chromosome 14 (HoxA) regions have not been previously reported. Thirty four of the 46 ACL rupture associated CNVRs were only identified by a single calling algorithm. Despite stringent quality control methods, CNV detection from SNP array data has inherent limitations and not all CNVs called by one algorithm are confirmed with PCR analysis. Some CNVRs may be false positives despite use of rigorous methodology. Two affected individuals with reported HoxD CNVR deletions identified by a single calling algorithm were not validated with qPCR suggesting some dogs had deletion CNVs falsely called at this location. By running multiple algorithms on a single data set and using a nominal significance threshold (max(T) permutation $P<0.05$), together with FDR correction for CNVs called by multiple algorithms, the reported CNVRs are as comprehensive and inclusive as possible to help detect small subtle genetic contribution to a polygenic disease which may have subsequently increased the chance of reporting false positives.

Other limitations of this study include lack of correction for sex and neutering, body condition, and population stratification. ParseCNV does not account for environmental factors in the CNV association analysis. Without consideration of relevant covariates in the analysis, associated CNVRs may reflect candidate loci that predispose Labrador Retrievers to other confounding factors such as obesity, or knee morphology. Future work should include information collected on sex, age neutered, and body condition and appropriately incorporate them into analysis to avoid effects that might confound the association analysis. Interpretation of our results must be considered in light of the fact that ACL rupture is a complex polygenic disease and that only a single breed of dog was studied. qPCR validation of other CNVRs, particularly the 2 CNVRs on chromosomes 6 and 24 that had overlapping areas and were confirmed by all 3 algorithms could have been performed.

This work sets the stage for further validation and replication of ACL rupture associated CNVs. Because ambiguity exists between detection of duplications/deletions associated with cases in overlapping regions using the different algorithms, there is a need to confirm the presence and direction of a deviation in copy number from the reference genome. Further investigation of the fine-scale architecture of the homeobox pathway CNVRs is warranted. Short-read WGS has increased in popularity as a tool for CNV validation [109, 110] and could be used as a validation step in the Labrador Retrievers used in this study. Over time short-read WGS costs have decreased making it a more readily available high-throughput technology that allows researchers to perform detailed analysis of whole genomes. Short-read WGS is advantageous for CNV validation because it enables the detection of CNV presence, CNV structure,
and determination of CNV breakpoints [111]. In addition, short-read WGS enables more detailed analysis of genomic loci identified by our GWAS and CNV analysis that will aid in revealing the genetic mechanisms influencing risk of ACL rupture. Analysis of a larger number of dogs for both femoral and tibial bony structural morphology would be helpful to examine the relationship between structural variation in genes that regulate skeletal patterning, limb morphology and associated risk of ACL rupture in more detail.

In conclusion, we identified 46 small effect CNVRs associated with ACL rupture in the Labrador Retriever in 39 unique regions of the genome. A total of 152 genes were located in associated CNVRs. Of these genes, 19 clustered into a significant homeobox biologic pathway. We provide evidence that homeobox genes could influence risk of ACL rupture in some dogs by influencing rTTW, an important feature of proximal tibial morphology. Going forward, the regions identified in this study should be validated using WGS to advance understanding of how CNVRs contribute to the genetic risk of ACL rupture. The relationship between associated CNVs and limb morphology also needs to be studied in more detail. Results of this work may aid in the creation of a genetic screening test for dogs at high genetic risk for ACL rupture and reveal further insight into the underlying biologic mechanisms that contribute to the etiology of the disease in both human beings and dogs.

Supporting information

S1 File. Programming commands used for PennCNV [50], QuantiSNP [52], and ParseCNV [53] for CNV calling and subsequent association analysis.
(PDF)

S2 File. Primers used for relative copy number qPCR.
(PDF)

S3 File. Examples of poor SNP clustering with the (a) standard cluster file compared to improved clustering with the (b) user-generated custom cluster file.
(PDF)

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References

1. Mather RC 3rd, Koenig L, Kocher MS, Dall TM, Gallo P, Scott DJ, et al. Societal and economic impact of anterior cruciate ligament tears. J Bone Joint Surg Am. 2013; 95: 1751–1759. https://doi.org/10.2106/JBJS.L.01705 PMID: 24088967

2. Olsson O, Isacsson A, Englund M, Frobell RB. Epidemiology of intra- and peri-articular structural injuries in traumatic knee joint hernarthrosis–data from 1145 consecutive knees with subacute MRI. Osteoarthr Cartil. 2016; 24: 1890–1897. https://doi.org/10.1016/j.joca.2016.06.006 PMID: 27374877

3. Mall NA, Chalmers PN, Moric M, Tanaka MJ, Cole BJ, Bach BR Jr, et al. Incidence and trends of anterior cruciate ligament reconstruction in the United States. Am J Sports Med. 2014; 42: 2363–2370. https://doi.org/10.1177/0363546514542796 PMID: 25086064

4. Risberg MA, Oiestad BE, Gunderson R, Aune AK, Engebretsen L, Culvenor A, et al. Changes in knee osteoarthritis, symptoms, and function after anterior cruciate ligament reconstruction: A 20 year prospective follow-up study. Am J Sports Med. 2016; 44: 1215–1224. https://doi.org/10.1177/0363546515626539 PMID: 26912292

5. McNair PJ, Marshall RN, Matheson JA. Important features associated with acute anterior cruciate ligament injury. N Z Med J. 1990; 103: 537–539. PMID: 2243642

6. Wojtys EM, Beaulieu ML, Ashton-Miller JA. New perspectives on ACL injury: On the role of repetitive sub-maximal knee loading in causing ACL fatigue failure. J Orthop Res. 2016; 34: 2059–2068. https://doi.org/10.1002/jor.23441 PMID: 27653237

7. Bourke HE, Salmon LJ, Waller A, Patterson V, Pinczewski LA. Survival of the anterior cruciate ligament graft and the contralateral ACL at a minimum of 15 years. Am J Sports Med. 2012; 40: 1985–1992. https://doi.org/10.1177/0363546512454414 PMID: 22869626

8. Kaynak M, Nijman F, van Meurs J, Reijman M, Meuffels DE. Genetic variants and anterior cruciate ligament rupture: A systematic review. Sports Med. 2017; 47: 1637–1650. https://doi.org/10.1007/s40279-017-0678-2 PMID: 28102489

9. Kim SK, Roos TR, Roos AK, Kleimeyer JP, Ahmed MA, Goodlin GT, et al. Genome-wide association screens for Achilles tendon and ACL tears and tendinopathy. PLoS ONE. 2017; 12: e0173810. https://doi.org/10.1371/journal.pone.0173810 PMID: 28379989

10. Goodlin GT, Roos AK, Roos TR, Hawkins C, Beach S, Baur S, et al. Applying personal genetic data to injury risk assessment in athletes. PLoS ONE. 2015; 10: e0122676. https://doi.org/10.1371/journal.pone.0122676 PMID: 25919592

11. Sutton KM, Bullock JM. Anterior cruciate ligament rupture: Differences between males and females. J Am Acad Orthop Surg. 2013; 21: 41–50. https://doi.org/10.5435/JAAOS-21-01-41 PMID: 23281470

12. Flynn RK, Pedersen CL, Birmingham TB, Kirkley A, Jackowski D, Fowler PJ. The familial predisposition toward tearing the anterior cruciate ligament: A case control study. Am J Sports Med. 2005; 33: 23–28. https://doi.org/10.1177/0363546504265678 PMID: 15610995

13. Kim SK, Roos TR, Roos AK, Kleimeyer JP, Ahmed MA, Goodlin GT, et al. Genome-wide association screens for Achilles tendon and ACL tears and tendinopathy. PLoS ONE. 2017; 12: e0170422. https://doi.org/10.1371/journal.pone.0170422 PMID: 28358823

14. Baker LA, Kirkpatrick B, Rosa GJ, Gianola D, Valente B, Sumner JP, et al. Genome-wide association analysis in dogs implicates 99 loci as risk variants for anterior cruciate ligament rupture. PLoS ONE. 2017; 12: e0173810. https://doi.org/10.1371/journal.pone.0173810 PMID: 28379989

15. Witsberger TH, Villamil JA, Schultz LG, Hahn AW, Cook JL. Prevalence of and risk factors for hip dysplasia and cranial cruciate ligament deficiency in dogs. J Am Vet Med Assoc. 2008; 232: 1818–1824. https://doi.org/10.2460/javma.232.12.1818 PMID: 18598150

16. Comerford EJ, Smith K, Hayashi K. Update on the aetiopathogenesis of canine cranial cruciate ligament disease. Vet Comp Orthop Traumatol. 2011; 24: 91–98. https://doi.org/10.3415/VCOT-10-04-0055 PMID: 21243176
17. Gregory MH, Capito N, Kuroki K, Stoker AM, Cook JL, Sherman SL. A review of translational animal models for knee osteoarthritis. Arthritis. 2012; 764621. https://doi.org/10.1155/2012/764621 PMID: 23326663

18. Chuang C, Ramaker MA, Kaur S, Csomas RA, Kroner KT, Bleedorn JA, et al. Radiographic risk factors for contralateral rupture in dogs with unilateral cranial cruciate ligament rupture. PLoS ONE. 2014; 9: e106389. https://doi.org/10.1371/journal.pone.0106389 PMID: 25254499

19. Webster KE, Feller JA, Leigh WB, Richmond AK. Younger patients are at increased risk for graft rupture and contralateral injury after anterior cruciate ligament reconstruction. Am J Sports Med. 2014; 42: 641–647. https://doi.org/10.1177/0363546513517540 PMID: 24451111

20. Panzer S, Augat P, Atzwanger J, Hergan K. 3-T MRI assessment of osteophyte formation in patients with unilateral anterior cruciate ligament injury and reconstruction. Skeletal Radiol. 2012; 41: 1597–1604. https://doi.org/10.1007/s00256-012-1445-y PMID: 22660837

21. Baird AE, Carter SD, Innes JF, Ollier WE, Short AD. Genetic basis of cranial cruciate ligament rupture (CCLR) in dogs. Connect Tissue Res. 2014; 55: 275–281. https://doi.org/10.3109/03008207.2014.910199 PMID: 24884544

22. Bleedorn JA, Greuel EN, Manley PA, Schaefer SL, Markel MD, Holzman G, et al. Synovitis in dogs with stable stifle joints and incipient cranial cruciate ligament rupture: A cross-sectional study. Vet Surg. 2011; 40: 531–536. https://doi.org/10.1111/j.1532-950X.2011.00841.x PMID: 21615432

23. Vasseur PB, Pool RR, Arnowczyk SP, Lau RE. Correlative biomechanical and histologic study of the cranial cruciate ligament in dogs. Am J Vet Res. 1985; 46: 1842–1854. PMID: 3901837

24. Whitehair JG, Vasseur PB, Willits NH. Epidemiology of cranial cruciate ligament rupture (CCLR) in dogs. Connect Tissue Res. 1985; 46: 1842–1854. PMID: 3901837

25. Doom M, de Bruin T, de Rooster H, van Bree H, Cox E. Immunopathological mechanisms in dogs with rupture of the cranial cruciate ligament. Vet Immunol Immunopathol. 2008; 125: 143–161. https://doi.org/10.1016/j.vetimm.2008.05.023 PMID: 18621423

26. Wilke VL, Conzemius MG, Kinghorn BP, Macrossan PE, Cai W, Rothschild MF. Inheritance of rupture of the cranial cruciate ligament in Newfoundlands. J Am Vet Med Assoc. 2006; 228: 61–64. https://doi.org/10.2460/javma.228.1.61 PMID: 16426167

27. Nielen AL, Janss LL, Knol BW. Heritability estimations for diseases, coat color, body weight, and height in a birth cohort of Boxers. Am J Vet Res. 2001; 62: 1198–1206. https://doi.org/10.2460/ajvr.2001.62.1198 PMID: 11497438

28. Baird AE, Carter SD, Innes JF, Ollier W, Short A. Genome-wide association study identifies genomic regions of association for cruciate ligament rupture in Newfoundland dogs. Anim Genet. 2014; 45: 542–549. https://doi.org/10.1111/age.12162 PMID: 24835129

29. Bailey JA, Eichler EE. Primate segmental duplications: Crucibles of evolution, diversity and disease. Nat Rev Genet. 2006; 7: 552–564. https://doi.org/10.1038/nrg1895 PMID: 16770338

30. Chen WK, Swartz JD, Rush LJ, Alvarez CE. Mapping DNA structural variation in dogs. Genome Res. 2009; 19: 500–509. https://doi.org/10.1101/gr.083741.108 PMID: 19015322

31. Alvarez CE, Akemy JM. Copy number variation in the domestic dog. Mamm Genome. 2012; 23: 144–163. https://doi.org/10.1007/s00335-011-9369-8 PMID: 22138850

32. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, Hunter DJ, et al. Finding the missing heritability of complex diseases. Nature. 2009; 461: 747–753. https://doi.org/10.1038/nature08494 PMID: 19812666

33. McCarroll SA, Altshuler DM. Copy-number variation and association studies of human disease. Nat Genet. 2007; 39: S37–S42. https://doi.org/10.1038/ng2080 PMID: 17597780

34. Beckmann JS, Estivill X, Antonarakis SE. Copy number variants and genetic traits: Closer to the resolution of phenotypic to genotypic variability. Nat Rev Genet. 2007; 8: 639–646. https://doi.org/10.1038/nrg2149 PMID: 17637735

35. Karlsson EK, Lindblad-Toh K. Leader of the pack: Gene mapping in dogs and other model organisms. Nat Rev Genet. 2008; 9: 713–725. https://doi.org/10.1038/nrg2362 PMID: 18714291

36. Nicholas TJ, Baker C, Eichler EE, Akemy JM. A high-resolution integrated map of copy number polymorphisms within and between breeds of the modern domesticated dog. BMC Genomics. 2011; 12: 1–10.

37. Molin AM, Berglund J, Webster MT, Lindblad-Toh K. Genome-wide copy number variant discovery in dogs using the CanineHD genotyping array. BMC Genomics. 2014; 15: 210. https://doi.org/10.1186/1471-2164-15-210 PMID: 24640994

38. Nicholas TJ, Cheng Z, Ventura M, Mealey K, Eichler EE, Akemy JM. The genomic architecture of segmental duplications and associated copy number variants in dogs. Genome Res. 2009; 19: 491–499. https://doi.org/10.1101/gr.084715.108 PMID: 19129542
39. Salmon Hillbertz NHC, Isaksson M, Karlsson EK, Hellmén E, Rosengren Pielberg G, Savolainen P, et al. Duplication of FGF3, FGF4, FGF19 and ORAOV1 causes hair ridge and predisposition to dermoid sinus in Ridgeback dogs. Nat Genet. 2007; 38: 1318–1320. https://doi.org/10.1038/ng.2007.4 PMID: 17906623

40. Saxena R, Voight BF, Lyssenko V, Burtt NP, de Bakker PI, Chen H, et al. Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. Science. 2007; 316: 1331–1336. https://doi.org/10.1126/science.1142358 PMID: 17463246

41. Perdry H, Dandine-Roulland C, Bandyopadhyay D, Kettner L. Package 'gaston': Genetic data handling (QC, GRM, LD, PCA) and linear mixed models. Version 1.5.3. ftp://cran.r-project.org/pub/R/web/packages/gaston/gaston.pdf.

42. Fuller MC, Hayashi K, Bruecker KA, Holsworth IG, Sutton JS, Kass PH, et al. Evaluation of the radio- graphic infrapatellar fat pad sign of the contralateral stifle joint as a risk factor for subsequent contralateral cranial cruciate ligament rupture in dogs with unilateral rupture: 96 cases (2006–2007). J Am Vet Med Assoc. 2014; 244: 328–338. https://doi.org/10.2460/javma.244.3.328 PMID: 24432965

43. Innes JF, Costello M, Barr FJ, Rudorf H, Barr AR. Radiographic progression of osteoarthritis of the canine stifle joint: A prospective study. Vet Radiol Ultrason. 2004; 45: 143–148. https://doi.org/10.1111/j.1740-8261.2004.04024.x PMID: 15072147

44. Reif U, Probst CW. Comparison of tibial plateau angles in normal and cranial cruciate deficient stifles of Labrador retrievers. Vet Surg. 2003; 32: 385–389. https://doi.org/10.1053/jvet.2003.50047 PMID: 12866002

45. Inauen R, Koch D, Bass M, Haessig M. Tibial tuberosity conformation as a risk factor for cranial cruciate ligament rupture in the dog. Vet Comp Orthop Traumatol. 2009; 22: 16–20. PMID: 19151865

46. Muir P, Schwartz Z, Malek S, Kreines A, Cabrera SY, Buote NJ, et al. Contralateral cruciate survival in dogs with unilateral non-contact cranial cruciate ligament rupture. PLoS ONE. 2011; 6: e25331. https://doi.org/10.1371/journal.pone.0025331 PMID: 21998650

47. Abel SB, Hammer DL, Shott S. Use of the proximal portion of the tibia for measurement of the tibial plateau angle in dogs. Am J Vet Res. 2003; 64: 1117–1123. https://doi.org/10.2460/ajvr.2003.64.1117 PMID: 13677389

48. Illumina. Infinium® Genotyping Data Analysis: A guide for analyzing Infinium genotyping data using GenomeStudio® Genotyping Module; 2010. Available from: http://www.illumina.com/content/dam/illumina-marketing/documents/services/technote_infinium_genotyping_data_analysis.pdf.

49. Illumina. Interpreting Infinium® Assay Data for Whole-Genome Structural Variation; Illumina offers a broad portfolio of DNA Analysis BeadChips for analyzing genotypes and structural variation. This document provided basic information about identifying copy number aberrations using Illumina whole-genome genotyping technology; 2010. Available from: http://www.illumina.com/Documents/products/technote_cytocentricanalysis.pdf.

50. Wang K, Li M, Hadley D, Liu R, Glesner J, Grant SF, et al. PennCNV: an integrated hidden Markov model designed for high-resolution copy number variation detection in whole-genome SNP genotyping data. Genome Res. 2007; 17: 1665–1674. https://doi.org/10.1101/gr.6861907 PMID: 17921354

51. Illumina. DNA Copy Number and Loss of Heterozygosity Analysis Algorithms: Detection of copy-number variant and chromosomal aberrations in GenomeStudio® software; 2014. Available from: http://www.illumina.com/documents/products/technote_dna_analysis_algorithms.pdf.

52. Colella S, Yau C, Taylor JM, Mirza G, Butler H, Clouston P, et al. QuantiSNP: An objective Bayes hidden-Markov model to detect and accurately map copy number variation using SNP genotyping data. Nucleic Acids Res. 2007; 35: 2013–2025. https://doi.org/10.1093/nar/gkm076 PMID: 17341461

53. Glesner JT, Li J, Hakonarson H. ParseCNV integrative copy number variation association software with quality tracking. Nucleic Acids Res. 2013; 41: e64. https://doi.org/10.1093/nar/gks1346 PMID: 23283001

54. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Series B Stat Methodol 1995; 57:289–300.

55. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009; 4: 44–57. https://doi.org/10.1038/nprot.2008.211 PMID: 19131956

56. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25: 402–408. https://doi.org/10.1066/meth.2001.1262 PMID: 11846609

57. Olsson M, Meadows JR, Truve K, Rosengren Pielberg G, Puppo F, Mauceli E, et al. A novel unstable duplication upstream of HAS2 predisposes to a breed-defining skin phenotype and a periodic fever syndrome in Chinese Shar-Pei dogs. PLoS Genet. 2011; 7; e1001332. https://doi.org/10.1371/journal.pgen.1001332 PMID: 21437276
58. Morris E, Lipowitz AJ. Comparison of tibial plateau angles in dogs with and without cranial cruciate ligament injuries. J Am Vet Med Assoc. 2001; 218: 363–366. https://doi.org/10.2460/javma.2001.218.363 PMID: 11201561

59. Lupski JR. Genomic rearrangements and sporadic disease. Nat Genet. 2007; 39: S43–S47. https://doi.org/10.1038/ng2084 PMID: 17597781

60. Orange JS, Glessner JT, Resnick E, Sullivan KE, Lucas M, Ferry B, et al. Genome-wide association identifies diverse causes of common variable immunodeficiency. J Allergy Clin Immunol. 2001; 127: 1360–1367.

61. Itsara A, Wu H, Smith JD, Nickerson DA, Romieu I, London SJ, et al. De novo rates and selection of large copy number variation. Genome Res. 2010; 20: 1469–1481. https://doi.org/10.1101/gr.107680.110 PMID: 20841430

62. Glessner JT, Bradfield JP, Wang K, Takahashi N, Zhang H, Sleiman PM, et al. A genome-wide study reveals copy number variants exclusive to childhood obesity cases. Am J Hum Genet. 2010; 87: 661–666. https://doi.org/10.1016/j.ajhg.2010.09.014 PMID: 20950786

63. Chettier R, Ward K, Albertsen HM. Endometriosis is associated with rare copy number variants. PLoS ONE. 2014; 9 e103968. https://doi.org/10.1371/journal.pone.0103968 PMID: 25083881

64. Tschopp P, Duboule D. A genetic approach to the transcriptional regulation of Hox gene clusters. Annu Rev Genet. 2011; 45: 145–166. https://doi.org/10.1146/annurev-genet-102209-163429 PMID: 22060042

65. Kuraku S, Meyer A. The evolution and maintenance of Hox gene clusters in vertebrates and the teleost-specific genome duplication. Int J Dev Biol. 2009; 53: 765–773. https://doi.org/10.1387/jdb.072533km PMID: 19557682

66. Tarchini B, Duboule D. Control of Hoxd genes’ collinearity during early limb development. Dev Cell. 2006; 10: 93–103. https://doi.org/10.1016/j.devcel.2005.11.014 PMID: 16399081

67. Montavon T, Thevenet L, Duboule D. Impact of copy number variations (CNVs) on long-range gene regulation at the HoxD locus. Proc Natl Acad Sci U S A. 2012; 109: 20204–20211. https://doi.org/10.1073/pnas.1217659109 PMID: 23134724

68. Kmita M, Tarchini B, Zakany J, Logan M, Tabin CJ, Duboule D. Early developmental arrest of mammalian limbs lacking HoxA/HoxD gene function. Nature. 2005; 435: 1113–1116. https://doi.org/10.1038/nature03648 PMID: 15973411

69. Goodman FR, Majewski F, Collins AL, Scambler PJ. A 117-kb microdeletion removing HOXD9-HOXD13 and EVX2 causes syndactyly. Am J Hum Genet. 2002; 70: 547–555. https://doi.org/10.1086/338921 PMID: 1217659109

70. Duerr FM, Duncan CG, Savicky RS, Park RD, Egger EL, Palmer RH. Risk factors for excessive tibial plateau angle in large-breed dogs with cranial cruciate ligament disease. J Am Vet Med Assoc. 2007; 231: 1688–1691. https://doi.org/10.2460/javma.231.11.1688 PMID: 18052804

71. Cook JL. Cranial cruciate ligament disease in dogs: Biology versus biomechanics. Vet Surg. 2010; 39: 270–277. https://doi.org/10.1111/j.1532-950X.2010.00653.x PMID: 20522028

72. Brown NP, Bertocci GE, Marcellin-Little DJ. Evaluation of varying morphological parameters on the biomechanics of a cranial cruciate ligament-deficient or intact canine stifle joint with a computer simulation model. Am J Vet Res. 2014; 75: 26–33. https://doi.org/10.2460/ajvr.75.1.26 PMID: 24370242

73. Fernandes MS, Pereira R, Andrade R, Vasta S, Pereira H, Pinheiro JP, et al. Is the femoral lateral condyle's bone morphology the trochlea of the ACL? Knee Surg Sports Traumatol Arthrosc. 2017; 25: 207–214. https://doi.org/10.1007/s00167-016-4159-1 PMID: 27161195

74. Koyama E, Yasuda T, Minugh-Purvis N, Kinumatsu T, Yallowitz AR, Wellik DM, et al. Hox11 genes establish synovial joint organization and phylogenetic characteristics in developing mouse zebrafish skeletal elements. Development. 2010; 137: 3795–3800. https://doi.org/10.1242/dev.053447 PMID: 20978074

75. Yueh YG, Gardner DP, Kappen C. Evidence for regulation of cartilage differentiation by the homeobox gene Hoxc-8. Proc Natl Acad Sci. 1998; 95: 9956–9961. https://doi.org/10.1073/pnas.95.17.9956 PMID: 9707582

76. Zakany J, Duboule D. The role of Hox genes during vertebrate limb development. Curr Opin Genet Dev. 2007; 17: 359–366. https://doi.org/10.1016/j.gde.2007.05.011 PMID: 17643733

77. Wilke V, Zaldívar-Lopez S, Ekenstedt K, Evans R, Conzemius M. Genotype influences risk of cranial cruciate ligament disease in the Newfoundland and Labrador Retriever breeds. J Vet Med Res. 2015; 2: 1028.

78. Axelsson E, Ratnakumar A, Arendt ML, Maqbool K, Webster MT, Perloski M, et al. The genomic signature of dog domestication reveals adaptation to a starch-rich diet. Nature. 2013; 495: 360–364. https://doi.org/10.1038/nature11837 PMID: 23354050
79. Arendt M, Fall T, Lindblad-Toh K, Axelsson E. Amylase activity is associated with AMY2B copy numbers in dog: implications for dog domestication, diet and diabetes. Anim Genet. 2014; 45: 716–722. https://doi.org/10.1111/age.12179 PMID: 24975239

80. Ollivier M, Tresset A, Bastian F, Lagoutte L, Axelsson E, Arendt ML, et al. Amy2B copy number variation reveals starch diet adaptations in ancient European dogs. R. Soc. Open Sci. 2016; 3: 160449. https://doi.org/10.1098/rsos.140449 PMID: 28018628

81. Pendleton AL, Shen F, Taravello AM, Emery S, Veeramah KR, Boyko AR, et al. Comparison of village dog and wolf genomes highlights the role of the neural crest in dog domestication. BMC Biology. 2018; 16: 64. https://doi.org/10.1186/s12915-018-0535-2 PMID: 29950181

82. Zhao E, Li J, Xie Y, Jin W, Zhang Z, Chen J, et al. Cloning and identification of a novel human RNPC3 gene that encodes a protein with two RRM domains and is expressed in the cell nucleus. Biochem Genet. 2003; 41: 315–323. https://doi.org/10.1023/b:bigi.0000006032.04031.d0 PMID: 14974681

83. Turunen JJ, Niemela EH, Verma B, Frilander MJ. The significant other: Splicing by the minor spliceosome. Wiley Interdiscip Rev RNA. 2013; 4: 61–76. https://doi.org/10.1002/wrna.1141 PMID: 23074130

84. Argente J, Flores R, Gutiérrez-Arumí A, Verma B, Martos-Moreno GÁ, Cusco I, et al. Defective minor spliceosome mRNA processing results in isolated familial growth hormone deficiency. EMBO Mol MED. 2014; 6: 299–306. https://doi.org/10.1002/emmm.201303573 PMID: 24480542

85. Yao J, Zeng K, Zhang M, Wei Q, Wang Y, Yang H, et al. OSBPL2-disrupted pigs recapitulate dual features of human hearing loss hypercholesterolaemia. J Gent Genomics. 2019; 46: 379–387. https://doi.org/10.1016/j.jgg.2019.06.006 PMID: 31451425

86. Briggs MD, Chapman KL. Pseudoachondroplasia and multiple epiphyseal dysplasia: Mutation review, molecular interactions, and genotype to phenotype correlations. Hum Mutat. 2002; 19: 465–478. https://doi.org/10.1002/humu.10066 PMID: 11968079

87. Jung WW, Balce GC, Cho JW, Jung SC, Hong SH, Song HR. COMP and COL9A3 mutations and their relationship to the pseudoachondroplasia phenotype. Int J Mol Med. 2010; 26: 885–891. https://doi.org/10.3892/ijmm_00000538 PMID: 21042783

88. Paassilta P, Lohiniva J, Annunen S, Bonaventure J, Merrer ML, Pai L, et al. COL9A3: A third locus for multiple epiphyseal dysplasia. Am J Hum Genet. 1999; 64: 1036–1044. https://doi.org/10.1086/302328 PMID: 10090888

89. Goldstein O, Guyon R, Kukekova A, Kuznetsova TN, Pearce-Kelling SE, Johnson J, et al. COL9A2 and COL9A3 mutations in canine autosomal recessive oculoskeletal dysplasia. Mammalian Genome. 2010; 21: 398–408. https://doi.org/10.1007/s00335-010-9276-4 PMID: 20686772

90. Carrig CB, MacMilan A, Brundage S, Pool RR, Morgan JP. Retinal dysplasia associated with skeletal abnormalities in Labrador Retrievers. J Am Vet Med Assoc. 1977; 170: 49–57. PMID: 830631

91. Wilke VL, Conzemius MC, Rothschild MF. SNP detection and association analysis of candidate genes for rupture of the cranial cruciate ligament in the dog. Anim Genet. 2005; 36: 519–521. https://doi.org/10.1111/j.1439-0442.2005.00980.x PMID: 16293131

92. Temwichitr J, Hazewinkel AW, van Hagen MA, Leegwater PAJ. Polymorphic microsatellite markers for genetic analysis of collagen genes in suspected collagenopathies in dogs. J Vet Med A. 2007; 54: 522–526. https://doi.org/10.1111/j.1439-0442.2007.00980.x PMID: 17931229

93. Tabor HK, Risch NJ, Myers RM. Candidate-gene approaches for studying complex genetic traits: practical considerations. Nat Rev Genet. 2002; 3: 391–397. https://doi.org/10.1038/nrg796 PMID: 11988764

94. Lamandé S, Bateman JF. Collagen VI disorders: Insights on form and function in the extracellular matrix and beyond. Matrix Biology. 2018; 71: 348–367. https://doi.org/10.1016/j.matbio.2017.12.008 PMID: 29277723

95. Sardone F, Traina F, Bondi A, Merlini L, Santi S, Maraldi NM, et al. Tendon extracellular matrix alterations in Ulrich congenital muscular dystrophy. Front Aging Neurosci. 2016; 8: 131. https://doi.org/10.3389/fnagi.2016.00131 PMID: 27375477

96. Izu Y, Ansorge HL, Zhang G, Soslowsky LJ, Bonaldo P, Chu ML, et al. Dysfunctional tendon collagen fibrillogenesis in collagen VI null mice. Matrix Biol. 2013; 30: 53–61.

97. Christensen E., Coles JM, Zelenski NA, Furman BD, Leddy HA, Zauscher S. Altered trabecular bone structure and delayed cartilage degeneration in the knees of collagen VI null mice. PLoS ONE. 2012; 7: e33397. https://doi.org/10.1371/journal.pone.0033397 PMID: 22448243

98. Alexopoulos LG, Youn I, Bonaldo P, Guilak F. Developmental and osteoarthritic changes in COL6A1-knockout mice: biomechanics of type VI collagen in the cartilage pericellular matrix. Arthritis Rheum. 2019; 60: 771–779.
99. Gu HY, Yang M, Guo J, Zhang C, Lin L, Liu Y, et al. Identification of biomarkers and pathological process of osteoarthritis: weighted gene co-expression network analysis. Front Physiol. 2019; 10: 275. https://doi.org/10.3389/fphys.2019.00275 PMID: 30941059

100. Fang Y, Wang P, Xia L, Bai S, Shen Y, Li Q, et al. Aberrantly hydroxymethylated differentially expressed genes and the associated protein pathways in osteoarthritis. Peer J. 2019; 7: e6425. https://doi.org/10.7717/peerj.6425 PMID: 30828485

101. De Smith AJ, Walters RG, Blakemore AI. Human genes involved in copy number variation: mechanisms of origin, functional effects and implications for disease. Cytogenet Genome Res. 2008; 123: 17–26. https://doi.org/10.1159/000184688 PMID: 19287135

102. Hastings PJ, Lupski JR, Rosenberg SM, Ira G. Mechanisms of change in gene copy number. Nat Rev Genet. 2008; 10: 551–564.

103. Tsuang DW, Millard SP, Ely B, Chi P, Wang K, Raskind WH, et al. The effect of algorithms on copy number variant detection. PLoS ONE. 2010; 5: e14456. https://doi.org/10.1371/journal.pone.0014456 PMID: 21209939

104. Pinto D, Darvishi K, Shi X, Rajan D, Rigler D, Fitzgerald T, et al. Comprehensive assessment of array-based platforms and calling algorithms for detection of copy number variants. Nat Biotechnol. 2011; 29: 512–520. https://doi.org/10.1038/nbt.1852 PMID: 21552272

105. Metzger J, Philipp U, Lopes MS, da Camara Machado A, Felicetti M, Silvestrelli M, et al. Analysis of copy number variants by three detection algorithms and their association with body size in horses. BMC Genomics. 2013; 14: 487. https://doi.org/10.1186/1471-2164-14-487 PMID: 23865711

106. Marenne G, Rodrı ´guez-Santiago B, Closas MG, Pe ´rez-Jurado L, Rothman N, Rico D, et al. Assessment of copy number variation using the Illumina Infinium 1M SNP-Array: A comparison of methodological approaches in the Spanish bladder cancer/EPICURO study. Hum Mutat. 2011; 32: 240–248. https://doi.org/10.1002/humu.21398 PMID: 21089066

107. Diskin SJ, Li M, Hou C, Yang S, Glessner J, Hakonarson H, et al. Adjustment of genomic waves in signal intensities from whole-genome SNP genotyping platforms. Nucleic Acids Res. 2008; 36: e126–e. https://doi.org/10.1093/nar/gkn556 PMID: 18784189

108. Berglund J, Nevalainen EM, Molin A, Perloski M, The LUPA Consortium, André C, et al. Novel origins of copy number variation in the dog genome. Genome Biology. 2012; 13: R73. https://doi.org/10.1186/gb-2012-13-8-r73 PMID: 22916802

109. Yoon S, Xuan Z, Makarov V, Ye K, Sebat J. Sensitive and accurate detection of copy number variants using read depth of coverage. Genome Res. 2009; 19: 1586–1592. https://doi.org/10.1101/gr.092981.109 PMID: 19657104

110. Duan J, Zhang JG, Deng HW, Wang YP. Comparative studies of copy number variation detection methods for next-generation sequencing technologies. PLoS ONE. 2013; 8: e59128. https://doi.org/10.1371/journal.pone.0059128 PMID: 23527109

111. Ceulemans S, van der Ven K, Del-Favero J. Targeted screening and validation of copy number variations. Methods Mol Biol. 2012; 838: 311–328. https://doi.org/10.1007/978-1-61779-507-7_15 PMID: 22228019