Genome-Wide Association Study in Dachshund: Identification of a Major Locus Affecting Intervertebral Disc Calcification

Mette Sloth Mogensen, Peter Karlskov-Mortensen, Helle Friis Proschowsky, Frode Lingaas, Anu Lappalainen, Hannes Lohi, Vibeke Frøkjær Jensen, and Merete Fredholm

From the Department of Basic Animal and Veterinary Sciences, Faculty of Life Sciences, University of Copenhagen, Grønnegårdsvej 3, 1870 Frederiksberg C, Denmark (Mogensen, Karlskov-Mortensen, Proschowsky, and Fredholm); the Department of Basic Sciences and Aquatic Medicine, Norwegian School of Veterinary Science, Oslo, Norway (Lingaas); the Equine and Small Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland (Lappalainen); the Department of Veterinary Biosciences, Department of Medical Genetics, Program in Molecular Medicine, Folkhälsan Research Center, University of Helsinki, Helsinki, Finland (Lohi); and the National Food Institute, Technical University of Denmark, Søborg, Denmark (Jensen).

Address correspondence to M. Fredholm at the address above, or e-mail: mf@life.ku.dk.

Abstract

Intervertebral disc calcification and herniation commonly affects Dachshund where the predisposition is caused by an early onset degenerative process resulting in disc calcification. A continuous spectrum of disc degeneration is seen within and among dog breeds, suggesting a multifactorial etiology. The number of calcified discs at 2 years of age determined by a radiographic evaluation is a good indicator of the severity of disc degeneration and thus serves as a measure for the risk of developing intervertebral disc herniation. The aim of the study was to identify genetic variants associated with intervertebral disc calcification in Dachshund through a genome-wide association (GWA) study. Based on thorough radiographic examinations, 48 cases with ≥ 6 disc calcifications or surgically treated for disc herniation and 46 controls with 0–1 disc calcifications were identified. GWA using the Illumina CanineHD BeadChip identified a locus on chromosome 12 from 36.8 to 38.6 Mb with 36 markers reaching genome-wide significance ($P_{\text{genome}} = 0.00001$–0.026). This study suggests that a major locus on chromosome 12 harbors genetic variations affecting the development of intervertebral disc calcification in Dachshund.

Key words: canine, genome-wide association study, intervertebral disc calcification and herniation

Introduction

Herniation of the intervertebral disc is a significant problem in dogs and a common cause of neurological dysfunction. The disease most commonly affects the Dachshund (Hansen 1952; Gage 1975; Priester 1976) where the relative risk is 10–12 times higher compared with all other breeds (Goggin et al. 1970; Priester 1976). The lifetime occurrence in the Dachshund is estimated to 19% with males and females equally affected (Ball et al. 1982). The intervertebral discs lie between the vertebral bodies, linking them together. They are complex structures consisting of 3 anatomical regions; an outer fibrous ring, the annulus fibrosus, which surrounds the gelatinous core, the nucleus pulposus, and the

cartilaginous endplates representing the cranial and caudal boundaries of the intervertebral disc. In the Dachshund and other hypochondroplastic breeds, the predisposition to intervertebral disc herniation is caused by an early degenerative process, which can result in disc calcification related to severe degeneration (Hansen 1952). The degeneration is preceded by early chondroid metaplasia emerging from the perinuclear zone and affecting the majority of the nucleus pulposus and perinuclear annulus fibrosus with profound matrix changes occurring within the first year of life (Hansen 1952; Ghosh et al. 1976). Herniation rarely occurs in dogs without disc calcifications while dogs with several calcifications are at particular high risk (Stigen 1996; Lappalainen et al. 2001). The number of

calcified discs at 2 years of age determined by a radiographic evaluation is a good indicator of the severe degeneration and is significantly correlated to the risk of developing intervertebral disc herniation (Jensen et al. 2008). A continuous spectrum of disc degeneration is seen within and among breeds suggesting a multifactorial etiology involving the cumulative effects of several genes and environmental factors (Ball et al. 1982). Severe disc degeneration with calcification has previously been shown highly heritable in Dachshund with heritability estimates of 0.47–0.87 (Jensen and Christensen 2000). The high heritability suggests that selection based on disc calcifications could induce a high response and change in population mean without changing the characteristic features of the breed (Jensen et al. 2008).

Disc herniation has long been a major health issue in the Danish Dachshund Club (DDC) and a breeding program has been initiated in order to decrease the occurrence of clinical disc herniation in the Dachshund population. The program is based on a radiographic examination of all dogs at 24–42 month of age where the number of calcified discs is determined. Since 2008, DDC has recommended that Dachshunds with ≥ 5 calcified discs were excluded from breeding. This screening prior to breeding has ensured a cohort of clinically well-characterized Dachshund allowing collection of samples for genetic studies. As a new initiative by the DDC a breeding value (see Materials and Methods) of the individual dog is calculated based on available information from all informative animals in a given pedigree and only dogs with a breeding value of above 100 (average in the breed) are recommended for breeding.

The extensive linkage disequilibrium and long haplotype blocks that characterizes the single dog breeds make the dog an excellent model to study complex diseases through the use of genome-wide association (GWA) studies. Due to the genetic homogeneity within dog breeds, fewer markers are required to identify disease association than compared with humans. This, in combination with the spontaneous occurrence of specific diseases in different breeds, indicating an enrichment of few genetic factors and the need for fewer samples, provide the dog with some advantages in studying genetic diseases (Sutter et al. 2004; Lindblad-Toh et al. 2005). The use of high density SNP arrays have already shown its strength in disease mapping in dogs and has opened doors toward a greater understanding of the genetic architecture of several complex diseases (Wood et al. 2009; Wilbe et al. 2010).

This study was performed within the LUPA project (http://www.eurolupa.org/) and it was aimed at identifying genetic variants associated with intervertebral disc calcification in Dachshunds via a GWA study.

Materials and Methods

Animals and diagnostic procedures

This study was confined to Dachshunds registered in the Danish Kennel Club. Inclusion criteria for sampling were based on radiographic examinations of intervertebral disc calcifications. The dogs were radiographed at age 24–42 month in right lateral recumbency and at least 5 lateral projections of each dog were performed covering the vertebral column from the second cervical vertebra to the third sacral bone. Every set of radiographs was evaluated technically and radiologically by the same radiologist to give the lowest possible test variation. The number of calcified discs (numeric score of 0–26) and location of the calcified disc were recorded according to the position in the cervical (C_1-T_1), thoracic (T_1-L_1), and lumbar regions (L_1-S_1) (Jensen and Ersbøll 2000). A breeding value was calculated on the basis of the radiographic examinations using a best linear unbiased prediction (BLUP) model (Henderson 1984) assuming a heritability of 0.5 and including age at radiology, sex, hair-variant, and year of evaluation as fixed effects (Kevin Byskov KB, personal communication). Information regarding hair variety (wire-haired, long-haired, and smooth-haired), size (standard, miniature, and rabbit), sex, age, and pedigree records was obtained from the Danish Kennel Club registry.

GWA mapping

Cases and controls for GWA studies were selected on the basis of stringent clinical criteria and pedigree information. Disease status was scored based on standard protocol for radiographic examinations. To ensure as distinct phenotypic classification as possible only dogs with either ≥ 6 disc calcifications or dogs that have undergone surgical treatment for disc herniation were classified as cases and dogs with ≤ 1 disc calcification were classified as controls. The distribution of disc calcifications among cases and controls is outlined in Table 1. In cases, the number of disc calcification varied from 6 to 15 with an average of 8.7. In addition, the study included 3 cases of long or smooth hair operated for disc herniation where the number of disc calcifications was not determined. In the control group, a total of 41 dogs had no disc calcifications while 5 dogs had one disc calcification. The breeding value for dogs classified as cases were on average 75 (from 51 to 92, standard deviation [SD] ± 8.9) and for dogs classified as controls on average 117 (110–127, SD ± 4.5).

Ethylenediaminetetraacetic acid–stabilized blood samples were collected with the owners consent by licensed veterinarians. Genomic DNA from 48 cases and 46 controls was isolated using standard methods (Miller et al. 1988). Genotyping was performed at Centre National de Génotypage, Every Cedex, France with the Illumina CanineHD BeadChip containing more than 170 000 markers placed on the CanFam2.0 reference sequence. Dogs were unrelated at parental level with few exceptions.

GWA was performed using PLINK (Purcell et al. 2007) and all markers were subject to strict quality control; only SNPs with a minor allele frequency >5%, a call rate of >90%, and in Hardy–Weinberg equilibrium in controls ($P = 0.05$) were included in subsequent analysis. All samples had less than 10% missing genotype calls. The threshold for

Table 1 Distribution of intervertebral disc calcifications among cases and controls

	Wire haired	Long haired	Smooth haired
Controls			
Number of disc calcifications			
0	27	10	4
1	1	3	1
Total	28	13	5
Cases			
Number of disc calcifications			
6	6		1
7	7		4
8	7	1	2
9	3		1
10	2		1
11		1	1
12	4		
13	2		
14	1		
15	1		
Operated for disc herniation		1	2
Total	33	3	12

Distribution of cases and controls according to hair-variant (wire-haired, long-haired, and smooth-haired) and individual scoring of disc calcifications determined by radiographic examinations (0 or 1 for controls and 6 to 15 or operated for herniation for cases).

genome-wide significance was set by a permutation test using 100 000 permutations. Multidimensional scaling (MDS) analysis with 4 dimensions to be extracted was carried out using PLINK (Purcell et al. 2007) and used to assess population stratification.

Results

Complete concordance between breeding value and number of disc calcifications were observed in our cohort. All dogs with ≥6 disc calcifications had an index below 100 and all dogs with 0 or 1 disc calcification had an index above 100. Looking at individual number of disc calcifications rather than breeding value allowed us to include 7 dogs for which breeding value was missing. Therefore, the GWA studies presented here were performed based on the individual scoring of disc calcifications.

To assess our sample for presence of stratification, we used MDS and produced a scatter plot for the first 2 dimensions as shown in Figure 1. Samples are represented by affection status and hair-variant and the MDS plot illustrates formation of 3 subclusters. The clustering pattern reveals the 3 hair-variants existing in the Dachshund population. The subcluster with smooth-haired dogs was predominantly cases (12/17), the subcluster with long-haired dogs was predominantly controls (13/16), whereas the subcluster with wire-haired dogs had an almost even mix (33/61 was cases). To control for confounding from the existing population structure, we performed the genome-wide scan in both the full sample material and separately in the wire-haired dogs.

To detect genetic variants affecting the development of canine intervertebral disc calcification, we used the GWA approach. In the full study sample, the genotyping success rate was >98% and >109 000 SNPs passed quality check. By analyzing 48 cases and 46 controls, we identified 2 loci on CFA12 reaching genome-wide significance after correcting raw P values for multiple hypothesis testing by permutation (see Figure 2A). The strongest association ($P_{genome} = 0.00011$) was detected within a region spanning from approximately 36.5–38.6 Mb. The second peak is a more narrow peak from 45.6 to 46 Mb where 3 SNPs in high linkage disequilibrium reach genome-wide significance ($P_{genome} = 0.00636$). Additionally, we identified a weaker signal on CFA3 with a single marker reaching genome-wide significance ($P_{genome} = 0.028$). The GWA study in wire-haired dogs included 33 cases and 28 controls. The strongest
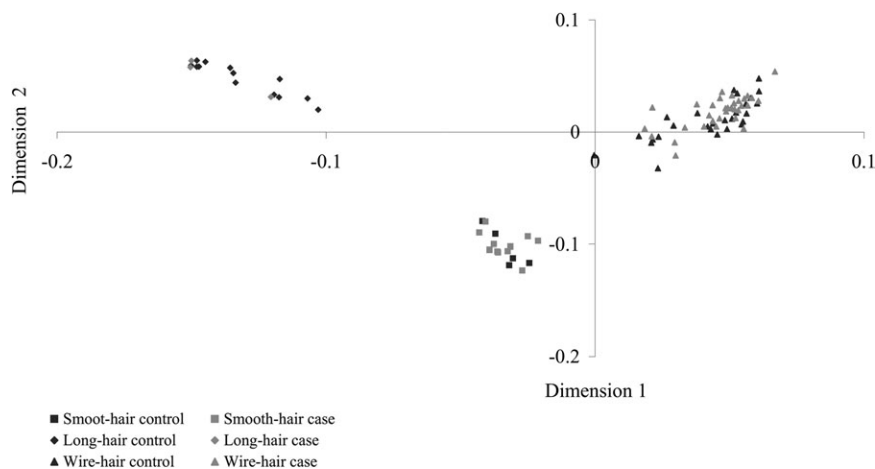

Figure 1. MDS plot of cases (gray symbols) and controls (black symbols) plotted for the first 2 dimensions. Each dot represents a specific dog. MDS findings indicate 3 distinct subclusters; smooth-haired subcluster (12 of 17 are cases), long-haired subcluster (13 of 16 are controls), wire-haired subcluster (33 of 61 are cases).
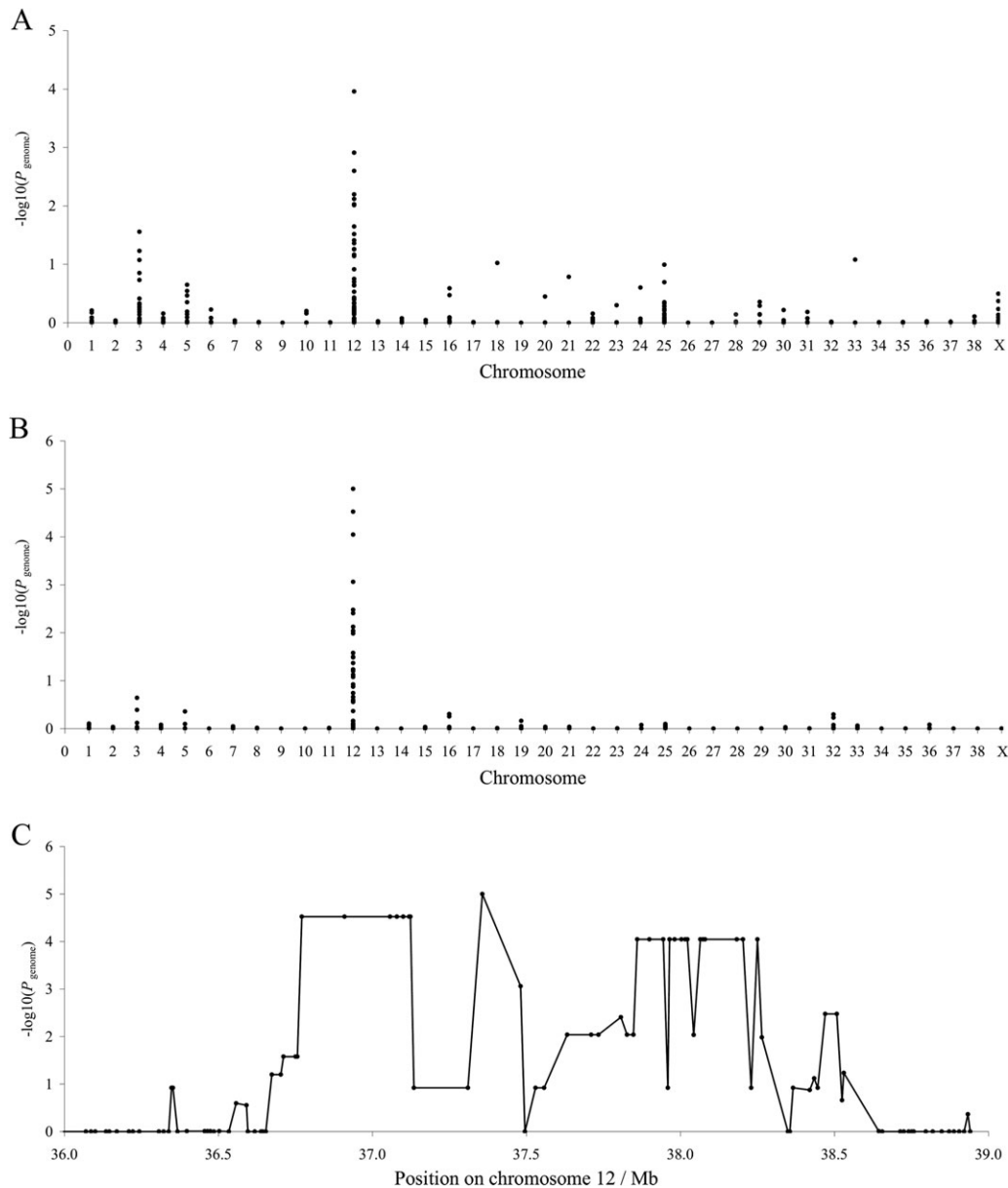
Figure 2. Manhatten plot showing *P* values corrected for multiple testing by 100 000 permutations ($-\log10(P_{genome}$ values)) on the *y* axis and the physical position of the markers sorted by chromosomal position on the *x* axis. (**A**) GWA of 48 cases and 46 controls showed a highly genome-wide significant association on CFA12 as well as a lower peak on CFA3. (**B**) Results of GWA analysis in wire-haired dogs (33 cases vs. 28 controls). One strongly associated region where found on CFA12. (**C**) Detailed view of the disease-associated region on CFA12. Position on CFA12 in megabases (Mb) is given on the *x* axis. *P* values on the *y* axis are from the GWA study in wire-haired dogs.

association was detected on CFA12 from 36.8 to 38.6 Mb and within this locus 36 markers reached genome-wide significance with *P* values in the range 0.00001–0.026 (Figure 2B). A close up on the CFA12 peak is shown in Figure 2C. Twenty-nine wire-haired cases are homozygous across all 36 markers. The most significant haplotype block within this region consists of 4 markers in a 35 kb region on CFA12 between 37 099 752 and 37 134 630 bp. The haplotype block distribution is outlined in Table 2. A second

smaller peak is present at 41.5–43.1 Mb with the most strongly associated marker having a P_{genome} of 0.0076. Because the CFA3 peak did not appear in the GWA study of the wire-haired dogs and because the strongest association was found in the 36–39 Mb region on CFA12, we intent to concentrate our initial studies on this particular region. The disease-associated region contains a total of 15 annotated protein-coding genes according to the corresponding human region; *FAM135A, C6orf57,*

Table 2 Distribution of the disease-associated haplotype (D) and the alternative haplotype (d) among cases and controls

	Total number	dd	Dd	DD
Cases	33	0	1	32
Controls	28	5	18	5

The haplotype block consists of 4 markers in a 35 kb region on CFA12 between 37 099 752 and 37 134 630 bp.

A1EAA2_CANFA, *B3GA2_CANFA*, *OGFRL1*, *RIMS1*, *KCNQ5*, *DDX43*, *C6orf221*, *OOEP_CANFA*, *C6orf150*, *MTO1*, *Q866G8_CANFA*, *SLC17A5*, and *CD109*.

Discussion

Our findings provide clear evidence for a region on CFA12 harboring genetic components affecting the development of disc calcification and thus the risk of herniation in Dachshund. Based on our results, variation in this disease-associated locus appears to be a major determinant in respect to development of disc calcification as no other strong candidate loci have been identified in this study. The haplotype sharing between cases resembles a recessive inheritance and supports the idea that a limited number of loci underlie disc calcification in Dachshund. In spite of very clear segregation of haplotypes in this locus, the disease is most likely affected by additional genetic and environmental factors. Genotyping of a larger cohort is needed to evaluate the impact of the 41.5–43.1 Mb CFA12 locus and to confirm or exclude the association to the locus on CFA3. The latter might represent a spurious association or a result of population structure because it disappears when the data set is adjusted for stratification.

This study highlights the importance of analyzing study samples for population structure as substructure easily can be present even within a single breed, thus leading to spurious associations. There is a clear stratification between different hair varieties in our Dachshund population; however, this is a logic consequence as Dachshund are not bred across hair varieties and therefore behave genetically as separate breeds.

Based on the annotation in publicly available databases, there are no genes within our candidate region with prior evidence of being involved in a biological relevant pathway. Because there are no obvious candidate genes, we have initiated resequencing of the genomic region of interest using the NimbleGen Sequence Capture technology and the Illumina platform. To evaluate the disease-causing potential of the polymorphisms identified through resequencing, we intent to genotype the variants in an independent collection of dogs from Denmark, Norway, and Finland and in unrelated dogs from a range of different breeds with a low prevalence of disc herniation.

The current arrays for GWA studies have focused on common variation in the genome. However, rarer variants with MAF < 5% and structural variations like copy number variants are also expected to contribute to the genetic basis of common diseases and efforts to detect these genetic variations should be included in future studies. The sample sizes used in this study is at the lower limit of having enough statistical power to detect disease-predisposing alleles in complex traits, but for a simple recessive trait, less than 20 cases and 20 controls is required (Lindblad-Toh et al. 2005). Our results are unambiguous with the genome-wide *P* values being more than 1000-fold stronger for the associated region than any other region in the genome. Hence, with the inheritance pattern observed in our population this study demonstrates that solid evidence for a phenotype–genotype association can be obtained even with a relatively small sample size collected in a closed breeding population of purebred dogs.

Human disease is characterized by marked genetic heterogeneity making genetic dissection challenging. Due to the reduced disease heterogeneity within the single dog breeds, our studies may be helpful in elucidating some of the genetic mechanism behind disc degeneration. In humans, studies of intervertebral disc degeneration have focused on candidate gene studies, but the etiology and disease pathogenesis of disc degeneration is still poorly understood (Videman et al. 2009). Because the assessment of disc degeneration in humans most often is performed by magnetic resonance imaging where it is difficult to evaluate disc calcifications, it is not possible to make a direct comparison between the etiology of the diseases in the 2 species. However, elucidating the biological processes involved in disc degeneration in dogs will undoubtedly shed light on the pathways relevant for the human condition.

In conclusion, we have identified a major locus on CFA12 affecting the development of intervertebral disc calcification in Dachshund. Because of the strong evidence for a disease-associated region identified by the GWA study, we believe that resequencing of the candidate region and a thorough follow-up on potential disease-causing variants will facilitate the identification of the disease-causing mutation in Dachshunds.

References

Ball MU, McGuire JA, Swain SA, Hoerlein BF. 1982. Patterns of occurrence of disk disease among registered Dachshunds. J Am Vet Med Assoc. 180:519–522.

Gage ED. 1975. Incidence of clinical disc disease in the dog. J Am Anim Hosp Assoc. 11:135–138.

Ghosh P, Taylor TK, Braund KG, Larsen LH. 1976. A comparative chemical and histochemical study of the chondrodystrophoid and non-chondrodystrophoid canine intervertebral disc. Vet Pathol. 13:414–427.

Goggin JE, Li AS, Franti CE. 1970. Canine intervertebral disc disease: characterization by age, sex, breed and anatomic site of involvement. Am J Vet Res. 31:1687–1692.

Hansen HJ. 1952. A pathologic-anatomical study on disc degeneration in dog. Acta Orthop Scand Suppl. 11:1–120.

Henderson CR. 1984. Application of linear models in animal breeding. Guelph (Canada): University of Guelph Press.

Jensen VF, Christensen KA. 2000. Inheritance of dics calcification in the dachshund. J Vet Med A Physiol Pathol Clin Med. 47:331–340.

Jensen VF, Beck S, Christensen KA, Arnbjerg J. 2008. Quantification of the association between intervertebral disk calcification and disk herniation in Dachshunds. J Am Vet Med Assoc. 223:1090–1095.

Jensen VF, Ersbøll AK. 2000. Mechanical factors affecting the occurrence of intervertebral disc calcification in the dachshund. A population study. J Vet Med A Physiol Pathol Clin Med. 47:283–296.

Lappalainen A, Norrgård M, Alm K, Snellman M, Laitinen O. 2001. Calcification of the intervertebral discs and curvature of the radius and ulna: a radiographic survey of Finnish miniature dachshunds. Acta Vet Scand. 42:229–236.

Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, Clamp M, Chang JL, Kulbokas EJ 3rd, Zody MC, et al. 2005. Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature. 438:803–819.

Miller SA, Dykes DD, Polesky HF. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16:1215.

Priester WA. 1976. Canine intervertebral disc disease—occurrence by age, breed, and sex among 8,117 cases. Theriogenology. 6:293–303.

Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, et al. 2007. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 81:559–575.

Stigen Ø. 1996. Calcification of intervertebral discs in the dachshund: a radiographic study of 115 dogs at 1 and 5 years of age. Acta Vet Scand. 37:229–237.

Sutter NB, Eberle MA, Parker HG, Pullar BJ, Kirkness EF, Kruglyak L, Ostrander EA. 2004. Extensive and breed-specific linkage disequilibrium in Canis familiaris. Genome Res. 14:2388–2396.

Videman T, Saarela J, Kaprio J, Nakki A, Levalahti E, Gill K, Peltonen L, Battié MC. 2009. Associations of 25 structural, degradative, and inflammatory candidate genes with lumbar disc desiccation, bulging, and height narrowing. Arthritis Rheum. 60:470–481.

Wilbe M, Jokinen P, Truvé K, Seppala EH, Karlsson EK, Biagi T, Hughes A, Bannasch D, Andersson G, Hansson-Hamlin H, et al. 2010. Genome-wide association mapping identifies multiple loci for a canine SLE-related disease complex. Nat Genet. 3:250–254.

Wood SH, Ke X, Nuttall T, McEwan N, Ollier WE, Carter SD. 2009. Genome-wide association analysis of canine atopic dermatitis and identification of disease related SNPs. Immunogenetics. 61: 765–772.