The Veterinary Journal 189 (2011) 220–226

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

# Expanded dog leukocyte antigen (DLA) single nucleotide polymorphism (SNP) genotyping reveals spurious class II associations

N. Safra [a,*], N.C. Pedersen [a], Z. Wolf [a], E.G. Johnson [a], H.W. Liu [a], A.M. Hughes [b], A. Young [a], D.L. Bannasch [a]

[a] School of Veterinary Medicine, University of California, Davis, USA
[b] Department of Animal Science, University of California, Davis, CA 95616, USA

ARTICLE INFO

*Keywords:*
Canine
Inherited disorders
Major histocompatibility complex (MHC)
Dog leukocyte antigen (DLA)
Disease associations

ABSTRACT

The dog leukocyte antigen (DLA) system contains many of the functional genes of the immune system, thereby making it a candidate region for involvement in immune-mediated disorders. A number of studies have identified associations between specific DLA class II haplotypes and canine immune hemolytic anemia, thyroiditis, immune polyarthritis, type I diabetes mellitus, hypoadrenocorticism, systemic lupus erythematosus-related disease complex, necrotizing meningoencephalitis (NME) and anal furunculosis. These studies have relied on sequencing approximately 300 bases of exon 2 of each of the DLA class II genes: DLA-DRB1, DLA-DQA1 and DLA-DQB1. In the present study, an association (odds ratio = 4.29) was identified by this method between Weimaraner dogs with hypertrophic osteodystrophy (HOD) and DLA-DRB1*01501.

To fine map the association with HOD, a genotyping assay of 126 coding single nucleotide polymorphisms (SNPs) from across the entire DLA, spanning a region of 2.5 Mb (3,320,000–5,830,000) on CFA12, was developed and tested on Weimaraners with HOD, as well as two additional breeds with diseases associated with DLA class II: Nova Scotia duck tolling retrievers with hypoadrenocorticism and Pug dogs with NME. No significant associations were found between Weimaraners with HOD or Nova Scotia duck tolling retrievers with hypoadrenocorticism and SNPs spanning the DLA region. In contrast, significant associations were found with NME in Pug dogs, although the associated region extended beyond the class II genes. By including a larger number of genes from a larger genomic region, a SNP genotyping assay was generated that provides coverage of the extended DLA region and may be useful in identifying and fine mapping DLA associations in dogs.



## Introduction

A large number of autoimmune disorders in dogs have been associated with certain alleles and haplotypes of the dog leukocyte antigen (DLA), which includes the canine major histocompatibility complex (MHC) class II genes DRB1, DQA1 and DQB1. This diverse group of diseases includes type I diabetes mellitus of the Samoyed dog and Cairn and Tibetan terriers (Kennedy et al., 2006a,c; Catchpole et al., 2008), immune-hemolytic anemia (Kennedy et al., 2006b), systemic lupus erythematosus-related disease complex (Wilbe et al., 2009) and hypoadrenocorticism (Hughes et al., 2010) in Nova Scotia duck tolling retrievers, anal furunculosis in German shepherd dogs (Kennedy et al., 2008), necrotizing meningoencephalitis (NME) of Pug dogs (Greer et al., 2010), autoimmune lymphocytic thyroiditis (Wilbe et al., 2010) and Vogt-Koyanagi-Harada-like syndrome of the Akita (Angles et al., 2005a).

These studies have taken a candidate gene approach to examine the classic DLA class II genes (DRB1, DQA1 and DQB1). The genes encode the molecules that associate to form the heterodimeric peptide binding cleft of the cells (macrophages, B cells and dendritic cells) that present exogenous antigens to $CD4^+$ T cells (Klein, 1986). Canine MHC association studies have focused on typing a portion (~300 bp) of the coding sequence of the second exon (which encodes the functional peptide binding domain) of each of the three classic class II genes (DRB1, DQA1 and DQB1); a fourth gene (DRA) is regarded as non-polymorphic (Wagner et al., 1995). The direct sequencing of this region (approximately 900 bases) conventionally yields the characterized DRB1/DQA1/DQB1 'alleles' and haplotypes (Kennedy, 2007; Kennedy et al., 2007a) and is utilized in the investigation of disease associations (Kennedy et al., 2007c).

Numerous reports have also described genetic associations between the human leukocyte antigen (HLA) classic class II genes (DRB1, DRA, DQA1 and DQB1) and rheumatoid arthritis (Fugger and Svejgaard, 2000; Holmdahl, 2000; Jawaheer et al., 2002; Coenen and Gregersen, 2009), type I diabetes (Lie et al., 1999a; Gorodezky et al., 2006; Ilonen and Hermann, 2010), multiple sclerosis (Svejgaard, 2008; Ramagopalan and Ebers, 2009; Dieudé et al., 2011) and autoimmune hypoadrenocorticism disease (Thomsen et al., 1975; Gombos et al., 2007; Falorni et al., 2008), as well as a range of other conditions.

* Corresponding author. Tel.: +1 530 7521317.
*E-mail address:* nsafra@ucdavis.edu (N. Safra).

1090-0233/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tvjl.2011.06.023

One postulated mechanism for the large number of associations between autoimmune diseases and the class II genes is allele-specific presentation of autoantigens to T cells (Raymond et al., 2005; Thorsby and Lie, 2005; Ettinger et al., 2006). According to this theory, differential presentation of antigens (which allows presentation of autoantigens) is a consequence of the amino acid sequence in the peptide-binding cleft of the heterodimer (Holmdahl, 2000; Weyand and Goronzy, 2000; Zanelli et al., 2000; Undlien et al., 2001; Ettinger et al., 2006).

However, since in multiple sclerosis, for example, all common HLA-DRB1 haplotypes are associated with either increased or decreased susceptibility, it is unlikely that the peptide sequence determines disease status (Ramagopalan and Ebers, 2009). So far, no mutations that can explain a mechanism for increased susceptibility have been identified in the classic MHC class II genes in humans (Holmdahl, 2000; Coenen and Gregersen, 2009; Ramagopalan and Ebers, 2009) or dogs (Kennedy et al., 2006a,b, 2007c, 2008; Barnes et al., 2009).

An association between DLA and hypertrophic osteodystrophy (HOD), an immune-mediated disorder in Weimaraner dogs, has been investigated by the authors since DLA involvement was implicated in many canine autoimmune diseases. HOD is a disease with specific breed predispositions that affects large breed dogs during the rapid growth stage (Bellah, 1993). Sick dogs exhibit swelling and pain in their legs, with reluctance to stand or walk. In addition to bone pain, there are variable general signs including fever, lethargy, depression and loss of appetite. The diagnosis of HOD is established by ruling out infectious osteomyelitis and by radiographic evidence of bone involvement (Watson et al., 1973; Grondalen, 1976; Woodard, 1982). The prognosis for severe cases is poor due to relapsing episodes and a low quality of life for affected puppies, often resulting in euthanasia.

The Weimaraner breed is susceptible to HOD and entire litters have been known to be affected (Woodard, 1982; Harrus et al., 2002). Although the cause of HOD is unknown, an inherited component to the disease is likely (Watson et al., 1973; Munjar et al., 1998; LaFond et al., 2002). An immune-mediated etiology has been suggested on the basis of a lack of response to conservative treatment with non-steroidal anti-inflammatory drugs concurrent with a positive response to immunosuppressive doses of corticosteroid treatment (Abeles et al., 1999; Harrus et al., 2002). DLA was considered as a candidate region in HOD of the Weimaraner because of the autoinflammatory nature of the disease. Autoinflammatory disorders are characterized by recurrent episodes of inflammation in the absence of infection or circulating autoantibodies (Farasat et al., 2008).

When the three DLA class II genes (DRB1, DQA1 and DQB1), were typed in Weimaraner dogs according to the method of Kennedy et al., (2007c), a significant association was identified between HOD and the DLA-DRB1∗01501 allele. Since no causative variants have been identified within the DRB1/DQA1/DQB1 genes so far (Fugger and Svejgaard, 2000; Ota et al., 2001; Gorodezky et al., 2006; Ramagopalan and Ebers, 2009; Eike et al., 2009), we 'fine mapped' the association between HOD and DLA in an attempt to uncover a causal variant.

To target the extended DLA region, we developed an expanded DLA-wide single nucleotide polymorphism (SNP) genotyping Sequenom assay with 126 SNPs across the 2.5 Mb DLA region. Using the DLA-wide assay, we then reanalyzed the association with Weimaraner HOD, as well as two previously published DRB1/DQA1/DQB1 disease associations: hypoadrenocorticism in the Nova Scotia duck tolling retriever (Hughes et al., 2010) and NME in Pug dogs (Greer et al., 2010). Differential results were obtained using the expanded DLA-wide panel, demonstrating the importance of confirming DLA sequenced haplotype associations.

## Materials and methods

### *DLA-wide Sequenom assay*

NCBI database coding (missense, nonsense and frameshift) SNPs[1] were mined across the extended DLA region (canine chromosome 12, CFA12:3.32–5.83 Mb) and the Assay Designer V.4.0 software (Sequenom) was used to develop an assay of 124 SNPs within 53 genes and two SNPs in non-coding sequence. To the best of our knowledge, these SNPs have not been used in previous DLA association studies. To represent the DLA class II genes that are conventionally represented in DLA association studies, six SNPs were selected from within the DRB1 gene, five SNPs from the DQA1 gene and two SNPs from the DQB1 gene. We also included two database SNPs representing missense mutations in DRA1, the fourth gene in the classic MHC II (Supplementary Table 1). The 126 SNPs were multiplexed into four Sequenom reaction wells of 37, 35, 33 and 21 SNPs, respectively (Supplementary Table 2).

Genotyping was performed using a Sequenom MassARRAY platform[2] at the Veterinary Genetics Laboratory, University of California, Davis, USA. Sample DNA was extracted from blood samples in ethylene diamine tetraacetic acid using a commercial kit (Puregene, Gentra Systems). Additional DNA was isolated from buccal swabs according to the method of Irion et al. (2003). PCR was performed in 5 μL volumes on an ABI 9700 Thermal Cycler (Applied Biosystems). Reactions and cycling conditions were performed according to the Sequenom iPLEX protocol for 96 well format using MassARRAY.

### *Study population*

Sets of samples from two breeds with previously reported DRB1/DQA1/DQB1 exon 2 associations were reused for genotyping using the Sequenom DLA-wide SNP panel, along with a set of samples from Weimaraners:

(1) Nova Scotia duck tolling retrievers with hypoadrenocorticism (34 cases and 34 controls) (Hughes et al., 2010): The Nova Scotia duck tolling retriever has an increased susceptibility for hypoadrenocorticism (Hughes et al., 2007). Hughes et al. (2010) reported an association (odds ratio, OR 2.8; 95% confidence interval, CI, 1.1–7.1; $P = 0.025$) between a specific haplotype (DLA-DRB1∗01502/DQA∗00601/DQB1∗02301) and hypoadrenocorticism in Nova Scotia duck tolling retrievers in the USA.
(2) Pug dogs with NME (40 cases and 42 controls) (Greer et al., 2010): Greer et al. (2009) reported the heritability of NME in Pug dogs and subsequently associated a haplotype (DRB1∗010011/DQA1∗00201/DQB1∗01501) with an increased risk of disease in the breed (OR 12.75) (Greer et al., 2010).
(3) Weimaraner dogs with HOD, first typed for DRB1/DQA1/DQB1 exon 2 regions (19 cases and 11 controls) and then genotyped using the DLA-wide panel (51 cases and 67 controls): Weimaraner samples were solicited using advertisements posted in the Weimaraner Club of America (WCA) magazine, on the WCA website, by direct communication with breeders and at WCA events. Medical records, including case history data and copies of radiographic images, were obtained for HOD cases and data were collected to determine the familial presentation of the disease in the breed, the influence of environmental factors and the health status of HOD cases as they mature. HOD cases were included on the basis of radiographic evidence of disease as evaluated by a board-certified veterinary radiologist (EGJ), systemic signs of hyperthermia and lethargy, lack of infection and response to treatment. The control group consisted of Weimaraners that did not show clinical signs of HOD. The sample set used for studying the DLA region included unrelated individuals to a third generation, based on a three-generation pedigree.

### *DRB1/DQA1/DQB1 exon 2 typing*

The second exons of the DRB1, DQA1 and DQB1 genes were typed by direct sequencing of genomic DNA from 19 cases of HOD in Weimaraners and 11 unaffected Weimaraners using locus-specific intronic primers, according to Kennedy et al. (2006a).

### *Haplotype assignment and statistical analysis*

DRB1/DQA1/DQB1 exon 2 haplotype determination was based on annotated sequences deposited in GenBank and the Immune Polymorphism Database (Robinson et al., 2005). Homozygous haplotypes identified at each locus were used to distinguish the heterozygous haplotypes. At least one parent of each animal was genotyped to allow verification of the assigned haplotype. Three-loci DRB1/DQA1/DQB1 super-haplotypes were identified according to Kennedy (2007) and Kennedy

[1] NCBI Data base SNPs: www.ncbi.nlm.nih.gov/projects/SNP/.
[2] SEQUENOM: www.sequenom.com/.

et al. (2007a,c). Haplotype frequencies in HOD cases and controls were compared by $\chi^2$ statistics. ORs with 95% CIs were calculated using a $2 \times 2$ contingency table (Bland and Altman, 2000).

*Statistical analysis for the DLA-wide Sequenom assay*

Association analysis by $\chi^2$ statistics was performed using Haploview software V.4.2 (Barrett et al., 2005). The four-gamete rule was used to define haplotype blocks and 1000 permutations were performed to correct for multiple-testing bias. The Pug dog NME *P* values were unresolved even with 10,000,000 permutations and the minimal obtainable (permuted) *P* value for the highest associated SNPs was $<1 \times 10^{-13}$ (Supplementary Table 3). ORs were generated using PLINK software (Purcell et al., 2007).

## Results

*Weimaraners and hypertrophic osteodystrophy*

*DRB1/DQA1/DQB1 exon 2 typing* – Nineteen Weimaraner HOD cases and 11 control Weimaraners were typed using the conventional method of sequencing exon 2 of DRB1/DQA1/DQB1. A significant association (OR 4.29; 95% CI 1.4–13.1; $P = 0.01$) between Weimaraner HOD and the DLA-DRB1∗01501 allele was identified (Table 1). A protective allele, DLA-DRB1∗01201, was also identified (OR 0.156; 95% CI 0.04–0.52; $P = 0.002$) (Table 1). These values are as high or higher than previously published class II associations (Kennedy et al., 2007c; Catchpole et al., 2008; Barnes et al., 2009). None of the seven other exon 2 alleles found in the Weimaraner sample set were associated with HOD. No significant association was observed between HOD and the three-locus super haplotypes in our data set (Table 2).

*DLA-wide single nucleotide polymorphism panel genotyping* – Fifty-one Weimaraner HOD cases and 67 control Weimaraner dogs were genotyped using the DLA-wide SNP panel. This larger sample set included the 19 HOD cases and 11 control Weimaraners that were studied by exon 2 sequencing. Of the 126 SNPs genotyped, 33 SNPs were not polymorphic in the Weimaraner data set and a total of 93 SNPs spanning the entire DLA region were analyzed (Supplementary Table 4). Even though the DRB1 gene was represented by five SNPs (Supplementary Table 4), no significant association was identified between the larger sample set of Weimaraner dogs with HOD and the DLA region (Supplementary Table 5).

*Nova Scotia duck tolling retrievers and hypoadrenocorticism*

*DLA-wide SNP panel genotyping* – Of the 126 SNPs assayed in the Nova Scotia duck tolling retriever sample set, 32 SNPs were not informative. A total of 94 SNPs spanning the entire DLA region were genotyped; the DRB1 gene was represented by six SNPs, DQB1 by two SNPs and DQA1 by five SNPs (Supplementary Table 4). No significant association ($P \leqslant 0.05$ based on 1000 permutations) was identified between Nova Scotia duck tolling retriever dogs with hypoadrenocorticism and the DLA region (Supplementary Table 6). As 10 additional cases and 17 additional controls became available, their genotypes were added to the analysis. No significant association was identified between the larger set of 44 hypoadrenocorticism cases and 51 control Nova Scotia duck tolling retrievers and DLA.

**Table 1**
DLA-DRB1∗01501 allele is associated with increased susceptibility to hypertrophic osteodystrophy (HOD) and DLA-DRB1∗01201 allele is associated with decreased susceptibility.

| DLA-DRB1 | HOD | Control | Odds ratio (95% confidence interval) | $\chi^2$ *P* value |
|---|---|---|---|---|
| 01501 | 27 | 8 | 4.29 (1.4–13.1) | 0.01 |
| 01201 | 6 | 12 | 0.156 (0.04–0.52) | 0.002 |
| 02001 | 5 | 2 | Not associated | |
| Total haplotypes | 38 | 22 | | |
| Total individuals | 19 | 11 | | |

*Necrotizing meningoencephalitis of Pug dogs*

*DLA-wide SNP panel genotyping* – Of the 126 SNPs assayed, 26 SNPs were not polymorphic in the Pug dog data set. A total of 100 SNPs spanning the extended DLA region were genotyped; the DRB1 gene was represented by six SNPs, DQB1 by two SNPs and DQA1 by five SNPs. Gene names, genomic locations of the SNPs and minor allele frequencies (MAF) are presented in Supplementary Table 4. SNPs located across the extended DLA region cfa12:3.32–5.83 (2.5 Mb) had significant associations with NME in Pug dogs (Supplementary Table 3); $\chi^2$ values 12.69 ($10^6$ permutations; $P = 0.0217$) to 53.37 ($10^6$ permutations; $P < 1 \times 10^{-13}$). The highest $\chi^2$ and lowest *P* values were assigned to SNP 22177967 (cfa12: 5.23 Mb), in the 3′ UTR of the DLA-DQA1 gene. The second highest $\chi^2$ value (52.2, $10^6$ permutations, $P < 1 \times 10^{-13}$) was assigned to SNP 22255769 (cfa12: 5.25 Mb), located near the 5′ UTR of the DLA-DQB1 gene, in agreement with the previously published DLA class II association (Greer et al., 2010).

When the significantly associated $\chi^2$ values were plotted by Mb position on CFA12 (Fig. 1), it was observed that highly significant $\chi^2$ values were assigned to SNPs located downstream of the DLA class II on cfa12. A $\chi^2$ value of 39.974 ($10^6$ permutations; $P = 3 \times 10^{-7}$) was assigned for SNP 8569523 located at the 3′ end of the typed region (cfa12: 5.76 Mb), within the DAXX gene.

## Discussion

We generated a DLA-wide SNP genotyping tool for fine mapping of an association between HOD in Weimaraners and DRB1 and tested two additional breeds with previously published DRB1/DQA1/DQB1 associations, Pug dogs with NME (Greer et al., 2010) and Nova Scotia duck tolling retrievers with hypoadrenocorticism (Hughes et al., 2010). Genotyping using the DLA-wide panel confirmed a strong association between NME in Pug dogs and the DLA; however, DLA associations in Nova Scotia duck tolling retrievers with hypoadrenocorticism and Weimaraners with HOD were not replicated using this expanded set of markers.

These results are in agreement with two previous publications: (1) a negative linkage analysis between Nova Scotia duck tolling retrievers with hypoadrenocorticism and the DLA class II region (Hughes et al., 2010); and (2) localization of NME of Pug dogs to the DLA region on cfa12 by a genome-wide association (GWA) study (Greer et al., 2010). Since the DLA-wide SNP panel assays the entire DLA region of 2.5 Mb containing 53 genes, including the MHC class II DRB1, DQA1 and DQB1 genes, for evidence of associations, it is unlikely that a true genetic association would be missed.

The reproducible association between DLA and NME of Pug dogs demonstrates that genetic associations can be identified using SNPs across the DLA. While SNPs from the entire DLA region were significantly associated with NME of Pug dogs, a plot of $\chi^2$ by physical position (Fig. 1) shows that the high $\chi^2$ values are not only observed around DLA class II, but remain high across the extended DLA region. This implies that the association with NME in the Pug dog might be located downstream of DLA class II, or outside the extended DLA region on CFA12.

**Table 2**
DLA class II exon 2 three-locus super-haplotypes determined in 30 hypertrophic osteodystrophy (HOD) cases and controls from the Weimaraner breed; no association was found between the identified super-haplotypes and HOD.

| DLA class II exon 2 super-haplotype | DRB1 | DQA1 | DQB1 | HOD | Control | Odds ratio |
|---|---|---|---|---|---|---|
| 1 | 01501 | 00601 | 01501 | 8 | 3 | Not associated |
| 2 | 01201 | 00401 | 01701 | 7 | 8 | |
| 3 | 01201 | 00401 | 01303 | 2 | 4 | |
| 4 | 02001 | 00401 | 01303 | 4 | 2 | |
| 5 | 01501 | 00401 | 01501 | 5 | 1 | |
| 6 | 01501 | 00601 | 02301 | 3 | 1 | |
| 7 | 01501 | 00401 | 01701 | 5 | 1 | |
| 8 | 01501 | 00301 | 01501 | 2 | 1 | |
| 9 | 01501 | 05011 | 00701 | 2 | 1 | |
| Total haplotypes | | | | 38 | 22 | |
| Total individuals | | | | 19 | 11 | |

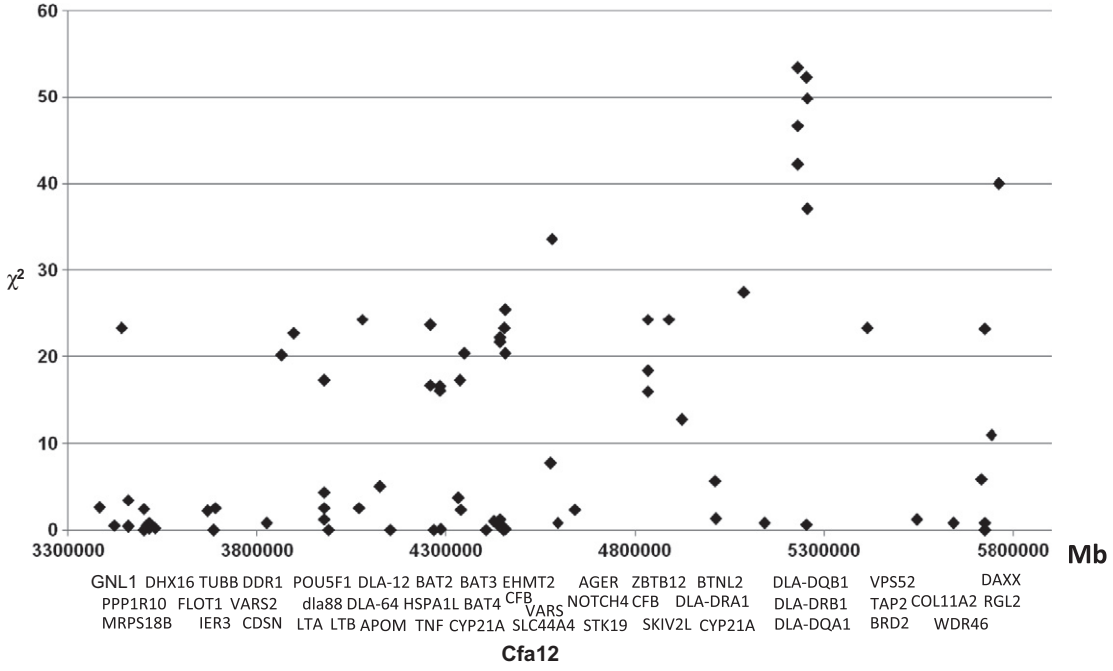


**Fig. 1.** Single nucleotide polymorphisms (SNPs) significantly associated with necrotizing meningoencephalitis (NME) of Pug dogs. $\chi^2$ values are plotted on the *Y* axis relative to the physical position (in Mb) on the *X* axis. Forty-six of the genes assayed using the DLA-wide SNP genotyping tool are presented (not to scale) along the *X* axis.

Since population stratification (different degrees of kinship between the case and control groups) could lead to spurious associations (Hinds et al., 2004; Wang, 2009; Wu et al., 2011), this should be considered as a potential cofounding factor in canine association studies. However, it is unlikely that the inconsistency in the DLA association results could be explained by population structure, because identical samples were used for the analyses. A possible explanation for the observed spurious associations could be the small number of DRB1/DQA1/DQB1 alleles in the Nova Scotia duck tolling retriever and Weimaraner breeds.

Although the DRB1/DQA1/DQB1 genes are highly polymorphic across different breeds of dogs, only limited numbers of alleles (typically 4–7 for each locus) have been identified within a range of breeds tested (Ollier et al., 2001; Angles et al., 2005b; Kennedy, 2007; Kennedy et al., 2006a–c, 2007a,b, 2008; Catchpole et al., 2008; Wilbe et al., 2009; Hughes et al., 2010). In Nova Scotia duck tolling retrievers, Hughes et al. (2010) identified seven different DRB1/DQA1/DQB1 haplotypes, comprising five DRB1 alleles, four DQA1 alleles and six DQB1 alleles. In Weimaraners, we identified nine different DRB1/DQA1/DQB1 haplotypes comprising three DRB1 alleles, four DQA1 alleles and five DQB1 alleles (Table 2). The DRB1/DQA1/DQB1 haplotypes reported by Kennedy et al. (2007a) comprised 100 DRB1 alleles, 60 DQB1 alleles and 26 DQA1 alleles identified across 80 different dog breeds. Out of 360 dogs from 25 breeds typed for the DRB1/DQA1/DQB1 alleles, Angles et al. (2005b) found that 40% were homozygous at the DRB1 site, 52% at the DQA1 and 44% at the DQB1 site.

Considering these findings, a significant statistical association within a breed population with a limited number of haplotypes should be considered cautiously and proof of a biological effect or linkage analysis should be sought as part of the validation of the statistical result. To illustrate that a spurious association is attainable in a breed with a small number of DLA alleles (conventionally tested), an association analysis was performed for Weimaraner sex (a trait determined by the presence or absence of the Y chromosome) and the DLA class II DRB1/DQA1/DQB1 alleles. DRB1 alleles associated with a 'high risk' of being a male (DRB1∗1501: OR 2.68; $P = 0.025$) as well as with a 'protective effect' (DRB1∗1201: OR 0.34; $P = 0.013$) were identified in this example of a spurious association. When the same association was performed using the DLA-wide SNP panel, no significant association was identified.

It is likely that the restricted number of DRB1/DQA1/DQB1 alleles and haplotypes do not segregate equally within a breed population due to inbreeding practices, use of popular sires, and/or genetic drift. Since both linkage disequilibrium (LD) and allelic content vary for different HLA haplotypes (Horton et al., 2008), this may be true for the DLA. The exon 2 'alleles' may represent different haplotypes that appear to be identical due to convergence (Seddon et al., 2010). When Seddon et al. (2010) sampled SNPs from the MHC class II region to evaluate canine cases and controls for type I diabetes, it was concluded that the published class II association with type I diabetes (Kennedy et al., 2006a) could be outside the DRB1/DQA1/DQB1 exon 2 region or within a region larger than just exon 2. It is also possible that associations are more complex than those involving a single haplotype (Seddon et al., 2010). Differentiation of each of these possibilities should be possible when genotyping SNPs across the extended DLA region for disease association studies.

We recognized that DRB1/DQA1/DQB1 exon 2 typing may give rise to spurious associations due to restricted numbers of haplotypes within breeds. This implies that exon 2 typing may not always be an informative tool when used to assess DLA diversity and that there is risk of misleading results due to focal low polymorphism. A DLA-wide SNP panel that spans the entire 2.5 Mb of the DLA region may present a more appropriate tool for the analysis of MHC diversity in pedigree dog populations.

The MHC region harbors the highest density of genes in the genome, yet most, if not all, of the genetic associations point to the class II classic genes in humans (Feder et al., 1996; Lie et al., 1999a,b; Undlien et al., 1999, 2001; Fugger and Svejgaard, 2000; Roudier, 2000; Weyand and Goronzy, 2000; Ota et al., 2001; Turner and Colbert, 2002; Raymond et al., 2005; Thorsby and Lie, 2005; Vandiedonck et al., 2005; Ettinger et al., 2006; Gorodezky et al., 2006; Falorni et al., 2008; Eike et al., 2009) and dogs (Ollier et al., 2001; Kennedy et al., 2006a–c, 2007c, 2008; Catchpole et al., 2008; Wilbe et al., 2009, 2010). In humans, an obstacle to localizing disease-predisposing genetic variants within the HLA is the strong LD typical of this region (Malfroy et al., 1997), presumably due to selection (Raymond et al., 2005).

LD or non-random association of alleles at adjacent HLA loci presents a difficulty in trying to establish which HLA locus is the primary disease-predisposing gene and which only appears to be associated because of LD to the primary associated gene (Feder et al., 1996). LD in the MHC is considered to be complex in comparison with other genomic regions demonstrated by events of deletions, duplications, and unequal crossing-over that have been documented within an inbred mouse strain (Kumánovics et al., 2002).

LD in dog breeds extends across 0.4–3.2 Mb depending on the size of the breed population and breed-specific history (Sutter et al., 2004; Lindblad-Toh et al., 2005). The long regions in LD are homozygous for breed-specific phenotypes and show a mosaic pattern of short ancestral haplotype blocks that are shared between different breeds (Karlsson et al., 2007). It is expected that disease association studies of DLA haplotypes will be affected by breed-specific long LD blocks as well as the restricted number of breed-specific DLA haplotypes, giving associations with linked but non-causative mutations (Seddon et al., 2010).

In humans, studies targeting the entire HLA region have led researchers to identify associations outside of the classic class II genes DRB1/DRA/DQA1/DQB1. For example, type 1 diabetes mellitus, an autoimmune disease with strong genetic associations to the HLA-DRB1/DQA1/DQB1 genes (Karlsson et al., 2007) has non-HLA risk loci identified by GWA (Wallace et al., 2010) and a meta-analysis of SNPs located across the extended HLA region enabled the identification of genetic associations to four potential causal loci (TCF19, POU5F1, CCHCR1 and PSORS1C1), all within a 56 kb region (Cheung et al., 2011). While it is presumed that strong LD within the HLA is a confounding factor in identifying associated genes outside DRB1/DRA/DQA1/DQB1, this could be overcome, as illustrated in this study, by utilizing novel analyzing algorithms for SNPs across the extended HLA region (Cheung et al., 2011).

## Conclusions

While human MHC studies advance to investigate the numerous MHC genes outside of the classic class II, progressing beyond the antigen-presenting peptide-cleft hypothesis, our data suggest that canine MHC association studies would benefit from the same approach. We generated a tool that allows DLA-wide investigation and could be utilized to identify disease associations within and outside DLA-II as well as evaluate DLA diversity in tested populations.

## Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriate influence of bias the content of the paper.

## Acknowledgements

This work was partially funded by Grant 1R21AI090277-01/NIH/NIAID, awarded to D.L. Bannasch, and by the Center for Companion Animal Health at the University of California, Davis.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tvjl.2011.06.023.

## References

Abeles, V., Harrus, S., Angles, J.M., Shalev, G., Aizenberg, I., Peres, Y., Aroch, I., 1999. Hypertrophic osteodystrophy in six Weimaraner puppies associated with systemic signs. Veterinary Record 145, 130–134.

Angles, J.M., Famula, T.R., Pedersen, N.C., 2005a. Uveodermatologic (VKH-like) syndrome in American Akita dogs is associated with an increased frequency of DQA1*00201. Tissue Antigens 66, 656–665.

Angles, J.M., Kennedy, L.J., Pedersen, N.C., 2005b. Frequency and distribution of alleles of canine MHC-II DLA-DQB1, DLA-DQA1 and DLA-DRB1 in 25 representative American Kennel Club breeds. Tissue Antigens 66, 173–184.

Barnes, A., O'Neill, T., Kennedy, L.J., Short, A.D., Catchpole, B., House, A., Binns, M., Fretwell, N., Day, M.J., Ollier, W.E., 2009. Association of canine anal furunculosis with TNFA is secondary to linkage disequilibrium with DLA-DRB1*. Tissue Antigens 73, 218–224.

Barrett, J.C., Fry, B., Maller, J., Daly, M.J., 2005. Haploview: Analysis and visualization of LD and haplotype maps. Bioinformatics 21, 263–265.

Bellah, J.R., 1993. Hypertrophic osteopathy. In: Bojrab, M.J., Smeak, D.D., Bloomberg, M.S. (Eds.), Disease Mechanisms in Small Animal Surgery, Second Ed. Lea & Febiger, Philadelphia, USA, pp. 858–864.

Bland, J.M., Altman, D.G., 2000. Statistics notes. The odds ratio. British Medical Journal 320, 1468.

Catchpole, B., Kennedy, L.J., Davison, L.J., Ollier, W.E., 2008. Canine diabetes mellitus: From phenotype to genotype. Journal of Small Animal Practice 49, 4–10.

Cheung, Y.H., Watkinson, J., Anastassiou, D., 2011. Conditional meta-analysis stratifying on detailed HLA genotypes identifies a novel type 1 diabetes locus around TCF19 in the MHC. Human Genetics 129, 161–176.

Coenen, M.J., Gregersen, P.K., 2009. Rheumatoid arthritis: A view of the current genetic landscape. Genes and Immunity 10, 101–111.

Dieudé, P., Boileau, C., Allanore, Y., 2011. Immunogenetics of systemic sclerosis. Autoimmunity Reviews 10, 282–290.

Eike, M.C., Becker, T., Humphreys, K., Olsson, M., Lie, B.A., 2009. Conditional analyses on the T1DGC MHC dataset: Novel associations with type 1 diabetes around HLA-G and confirmation of HLA-B. Genes and Immunity 10, 56–67.

Ettinger, R.A., Papadopoulos, G.K., Moustakas, A.K., Nepom, G.T., Kwok, W.W., 2006. Allelic variation in key peptide-binding pockets discriminates between closely related diabetes-protective and diabetes-susceptible HLA-DQB1*06 alleles. Journal of Immunology 176, 1988–1998.

Falorni, A., Brozzetti, A., Torre, D.L., Tortoioli, C., Gambelunghe, G., 2008. Association of genetic polymorphisms and autoimmune Addison's disease. Expert Review of Clinical Immunology 4, 441–456.

Farasat, S., Aksentijevich, I., Toro, J.R., 2008. Autoinflammatory diseases: Clinical and genetic advances. Archives of Dermatology 144, 392–402.

Feder, J.N., Gnirke, A., Thomas, W., Tsuchihashi, Z., Ruddy, D.A., Basava, A., Dormishian, F., Domingo, R. Jr., Ellis, M.C., Fullan, A., Hinton, L.M., Jones, N.L., Kimmel, B.E., Kronmal, G.S., Lauer, P., Lee, V.K., Loeb, D.B., Mapa, F.A., McClelland, E., Meyer, N.C., Mintier, G.A., Moeller, N., Moore, T., Morikang, E., Prass, C.E., Quintana, L., Starnes, S.M., Schatzman, R.C., Brunke, K.J., Drayna, D.T., Risch, N.J., Bacon, B.R., Wolff, R.K., 1996. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nature Genetics 13, 399–408.

Fugger, L., Svejgaard, A., 2000. HLA-DR4 and rheumatoid arthritis: Studies in mice and men. Arthritis Research 2, 208–211.

Klein, J., 1986. Natural History of the Major Histocompatibility Complex. John Wiley & Sons, New York, USA, 775 pp.

Gombos, Z., Hermann, R., Kiviniemi, M., Nejentsev, S., Reimand, K., Fadeyev, V., Peterson, P., Uibo, R., Ilonen, J., 2007. Analysis of extended human leukocyte antigen haplotype association with Addison's disease in three populations. European Journal of Endocrinology 157, 757–761.

Gorodezky, C., Alaez, C., Murguía, A., Rodríguez, A., Balladares, S., Vazquez, M., Flores, H., Robles, C., 2006. HLA and autoimmune diseases: Type 1 diabetes (T1D) as an example. Autoimmunity Reviews 5, 187–194.

Greer, K.A., Schatzberg, S.J., Porter, B.F., Jones, K.A., Famula, T.R., Murphy, K.E., 2009. Heritability and transmission analysis of necrotizing meningoencephalitis in the Pug. Research in Veterinary Science 86, 438–442.

Greer, K.A., Wong, A.K., Liu, H., Famula, T.R., Pedersen, N.C., Ruhe, A., Wallace, M., Neff, M.W., 2010. Necrotizing meningoencephalitis of Pug dogs associates with dog leukocyte antigen class II and resembles acute variant forms of multiple sclerosis. Tissue Antigens 76, 110–118.

Grondalen, J., 1976. Metaphyseal osteopathy (hypertrophic osteodystrophy) in growing dogs: A clinical study. Journal of Small Animal Practice 17, 721–735.

Harrus, S., Waner, T., Aizenberg, J., Safra, N., Mosenco, A., Radoshitsky, M., Bark, H., 2002. Development of hypertrophic osteodystrophy and antibody response in a litter of vaccinated Weimaraner puppies. Journal of Small Animal Practice 43, 27–31.

Hinds, D.A., Stokowski, R.P., Patil, N., Konvicka, K., Kershenobich, D., Cox, D.R., Ballinger, D.G., 2004. Matching strategies for genetic association studies in structured populations. American Journal of Human Genetics 74, 317–325.

Holmdahl, R., 2000. Association of MHC and rheumatoid arthritis: Why is rheumatoid arthritis associated with the MHC genetic region? An introduction. Arthritis Research 2, 203–204.

Horton, R., Gibson, R., Coggill, P., Miretti, M., Allcock, R.J., Almeida, J., Forbes, S., Gilbert, J.G., Halls, K., Harrow, J.L., Hart, E., Howe, K., Jackson, D.K., Palmer, S., Roberts, A.N., Sims, S., Stewart, C.A., Traherne, J.A., Trevanion, S., Wilming, L., Rogers, J., de Jong, P.J., Elliott, J.F., Sawcer, S., Todd, J.A., Trowsdale, J., Beck, S., 2008. Variation analysis and gene annotation of eight MHC haplotypes: The MHC Haplotype Project. Immunogenetics 60, 1–18.

Hughes, A.M., Jokinen, P., Bannasch, D.L., Lohi, H., Oberbauer, A.M., 2010. Association of a dog leukocyte antigen class II haplotype with hypoadrenocorticism in Nova Scotia duck tolling retrievers. Tissue Antigens 75, 684–690.

Hughes, A.M., Nelson, R.W., Famula, T.R., Bannasch, D.L., 2007. Clinical features and heritability of hypoadrenocorticism in Nova Scotia duck tolling retrievers: 25 cases (1994–2006). Journal of the American Veterinary Medical Association 231, 407–412.

Ilonen, J., Hermann, R., 2010. Novel gene associations in type 1 diabetes. Current Diabetes Reports 10, 338–344.

Irion, D.N., Schaffer, A.L., Famula, T.R., Eggleston, M.L., Hughes, S.S., Pedersen, N.C., 2003. Analysis of genetic variation in 28 dog breed populations with 100 microsatellite markers. Journal of Heredity 94, 81–87.

Jawaheer, D., Li, W., Graham, R.R., Chen, W., Damle, A., Xiao, X., Monteiro, J., Khalili, H., Lee, A., Lundsten, R., Begovich, A., Bugawan, T., Erlich, H., Elder, J.T., Criswell, L.A., Seldin, M.F., Amos, C.I., Behrens, T.W., Gregersen, P.K., 2002. Dissecting the genetic complexity of the association between human leukocyte antigens and rheumatoid arthritis. American Journal of Human Genetics 71, 585–594.

Karlsson, E.K., Baranowska, I., Wade, C.M., Salmon Hillbertz, N.H., Zody, M.C., Anderson, N., Biagi, T.M., Patterson, N., Pielberg, G.R., Kulbokas, E.J. 3rd., Comstock, K.E., Keller, E.T., Mesirov, J.P., von Euler, H., Kämpe, O., Hedhammar, A., Lander, E.S., Andersson, G., Andersson, L., Lindblad-Toh, K., 2007. Efficient mapping of Mendelian traits in dogs through genome-wide association. Nature Genetics 39, 1321–1328.

Kennedy, L.J., Davison, L.J., Barnes, A., Short, A.D., Fretwell, N., Jones, C.A., Lee, A.C., Ollier, W.E., Catchpole, B., 2006a. Identification of susceptibility and protective major histocompatibility complex haplotypes in canine diabetes mellitus. Tissue Antigens 68, 467–476.

Kennedy, L.J., Barnes, A., Ollier, W.E., Day, M.J., 2006b. Association of a common dog leucocyte antigen class II haplotype with canine primary immune-mediated haemolytic anaemia. Tissue Antigens 68, 502–508.

Kennedy, L.J., Quarmby, S., Happ, G.M., Barnes, A., Ramsey, I.K., Dixon, R.M., Catchpole, B., Rusbridge, C., Graham, P.A., Hillbertz, N.S., Roethel, C., Dodds, W.J., Carmichael, N.G., Ollier, W.E., 2006c. Association of canine hypothyroidism with a common major histocompatibility complex DLA class II allele. Tissue Antigens 68, 82–86.

Kennedy, L.J., 2007. 14th International HLA and immunogenetics workshop: Report on joint study on canine DLA diversity. Tissue Antigens 69, 269–271.

Kennedy, L.J., Barnes, A., Short, A., Brown, J.J., Lester, S., Seddon, J., Fleeman, L., Francino, O., Brkljacic, M., Knyazev, S., Happ, G.M., Ollier, W.E., 2007a. Canine DLA diversity: 1. New alleles and haplotypes. Tissue Antigens 69, 272–288.

Kennedy, L.J., Barnes, A., Short, A., Brown, J.J., Lester, S., Seddon, J., Happ, G.M., Ollier, W.E., 2007b. Canine DLA diversity: 2. Family studies. Tissue Antigens 69, 289–291.

Kennedy, L.J., Barnes, A., Short, A., Brown, J.J., Seddon, J., Fleeman, L., Brkljacic, M., Happ, G.M., Catchpole, B., Ollier, W.E., 2007c. Canine DLA diversity: 3. Disease studies. Tissue Antigens 69, 292–296.

Kennedy, L.J., O'Neill, T., House, A., Barnes, A., Kyöstilä, K., Innes, J., Fretwell, N., Day, M.J., Catchpole, B., Lohi, H., Ollier, W.E., 2008. Risk of anal furunculosis in German shepherd dogs is associated with the major histocompatibility complex. Tissue Antigens 71, 51–56.

Kumánovics, A., Madan, A., Qin, S., Rowen, L., Hood, L., Fischer Lindahl, K., 2002. QUOD ERAT FACIENDUM: Sequence analysis of the H2-D and H2-Q regions of 129/SvJ mice. Immunogenetics 54, 479–489.

LaFond, E., Breur, G.J., Austin, C.C., 2002. Breed susceptibility for developmental orthopedic diseases in dogs. Journal of the American Animal Hospital Association 38, 467–477.

Lie, B.A., Todd, J.A., Pociot, F., Nerup, J., Akselsen, H.E., Joner, G., Dahl-Jørgensen, K., Rønningen, K.S., Thorsby, E., Undlien, D.E., 1999a. The predisposition to type 1 diabetes linked to the human leukocyte antigen complex includes at least one non-class II gene. American Journal of Human Genetics 64, 793–800.

Lie, B.A., Sollid, L.M., Ascher, H., Ek, J., Akselsen, H.E., Rønningen, K.S., Thorsby, E., Undlien, D.E., 1999b. A gene telomeric of the HLA class I region is involved in predisposition to both type 1 diabetes and coeliac disease. Tissue Antigens 54, 162–168.

Lindblad-Toh, K., Wade, C.M., Mikkelsen, T.S., et al., 2005. Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature 438, 803–819.

Malfroy, L., Roth, M.P., Carrington, M., Borot, N., Volz, A., Ziegler, A., Coppin, H., 1997. Heterogeneity in rates of recombination in the 6-Mb region telomeric to the human major histocompatibility complex. Genomics 43, 226–231.

Munjar, T.A., Austin, C.C., Breur, G.J., 1998. Comparison of risk factors for hypertrophic osteodystrophy, craniomandibular osteopathy and canine distemper virus infection. Veterinary and Comparative Orthopaedics and Traumatology 11, 42–48.

Ollier, W.E., Kennedy, L.J., Thomson, W., Barnes, A.N., Bell, S.C., Bennett, D., Angles, J.M., Innes, J.F., Carter, S.D., 2001. Dog MHC alleles containing the human RA shared epitope confer susceptibility to canine rheumatoid arthritis. Immunogenetics 53, 669–673.

Ota, M., Katsuyama, Y., Kimura, A., Tsuchiya, K., Kondo, M., Naruse, T., Mizuki, N., Itoh, K., Sasazuki, T., Inoko, H., 2001. A second susceptibility gene for developing rheumatoid arthritis in the human MHC is localized within a 70-kb interval telomeric of the TNF genes in the HLA class III region. Genomics 71, 263–270.

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A., Bender, D., Maller, J., Sklar, P., de Bakker, P.I., Daly, M.J., Sham, P.C., 2007. PLINK: A tool set for whole-genome association and population-based linkage analyses. American Journal of Human Genetics 81, 559–575.

Ramagopalan, S.V., Ebers, G.C., 2009. Multiple sclerosis: Major histocompatibility complexity and antigen presentation. Genome Medicine 1, 105.

Raymond, C.K., Kas, A., Paddock, M., Qiu, R., Zhou, Y., Subramanian, S., Chang, J., Palmieri, A., Haugen, E., Kaul, R., Olson, M.V., 2005. Ancient haplotypes of the HLA class II region. Genome Research 15, 1250–1257.

Robinson, J., Waller, M.J., Stoehr, P., Marsh, S.G., 2005. IPD – The immuno polymorphism database. Nucleic Acids Research 33, D523–D526.

Roudier, J., 2000. Association of RA with HLA-DR4: The role of repertoire selection. Arthritis Research 2, 217–220.

Seddon, J.M., Berggren, K.T., Fleeman, L.M., 2010. Evolutionary history of DLA class II haplotypes in canine diabetes mellitus through single nucleotide polymorphism genotyping. Tissue Antigens 75, 218–226.

Sutter, N.B., Eberle, M.A., Parker, H.G., Pullar, B.J., Kirkness, E.F., Kruglyak, L., Ostrander, E.A., 2004. Extensive and breed-specific linkage disequilibrium in *Canis familiaris*. Genome Research 14, 2388–2396.

Svejgaard, A., 2008. The immunogenetics of multiple sclerosis. Immunogenetics 60, 275–286.

Thomsen, M., Platz, P., Andersen, O.O., Christy, M., Lyngsøoe, J., Nerup, J., Rasmussen, K., Ryder, L.P., Nielsen, L.S., Svejgaard, A., 1975. MLC typing in juvenile diabetes mellitus and idiopathic Addison's disease. Immunological Reviews 22, 125–147.

Thorsby, E., Lie, B.A., 2005. HLA associated genetic predisposition to autoimmune diseases: Genes involved and possible mechanisms. Transplant Immunology 14, 175–182.

Turner, M.J., Colbert, R.A., 2002. HLA-B27 and pathogenesis of spondyloarthropathies. Current Opinion in Rheumatology 14, 367–372.

Undlien, D.E., Kockum, I., Rønningen, K.S., Lowe, R., Saanjeevi, C.B., Graham, J., Lie, B.A., Akselsen, H.E., Lernmark, A., Thorsby, E., 1999. HLA associations in type 1 diabetes among patients not carrying high-risk DR3-DQ2 or DR4-DQ8 haplotypes. Tissue Antigens 54, 543–551.

Undlien, D.E., Lie, B.A., Thorsby, E., 2001. HLA complex genes in type 1 diabetes and other autoimmune diseases. Which genes are involved? Trends in Genetics 17, 93–100.

Vandiedonck, C., Giraud, M., Garchon, H.J., 2005. Genetics of autoimmune myasthenia gravis: The multifaceted contribution of the HLA complex. Journal of Autoimmunity 25, 6–11.

Wagner, J.L., DeRose, S.A., Burnett, R.C., Storb, R., 1995. Nucleotide sequence and polymorphism analysis of canine DRA cDNA clones. Tissue Antigens 45, 284–287.

Wallace, C., Smyth, D.J., Maisuria-Armer, M., Walker, N.M., Todd, J.A., Clayton, D.G., 2010. The imprinted DLK1-MEG3 gene region on chromosome 14q32. 2 Alters susceptibility to type 1 diabetes. Nature Genetics 42, 68–71.

Wang, K., 2009. Testing for genetic association in the presence of population stratification in genome-wide association studies. Genetic Epidemiology 33, 637–645.

Watson, A.D.J., Blair, R.C., Farrow, B.R.H., Baird, J.D., Cooper, H.L., 1973. Hypertrophic osteodystrophy in the dog. Australian Veterinary Journal 49, 433–439.

Weyand, C., Goronzy, J., 2000. HLA polymorphisms in phenotypic variants of rheumatoid arthritis. Arthritis Research 2, 212–216.

Wilbe, M., Jokinen, P., Hermanrud, C., Kennedy, L.J., Strandberg, E., Hansson-Hamlin, H., Lohi, H., Andersson, G., 2009. MHC class II polymorphism is associated with a canine SLE-related disease complex. Immunogenetics 61, 557–564.

Wilbe, M., Sundberg, K., Hansen, I.R., Strandberg, E., Nachreiner, R.F., Hedhammar, A., Kennedy, L.J., Andersson, G., Björnerfeldt, S., 2010. Increased genetic risk or protection for canine autoimmune lymphocytic thyroiditis in Giant Schnauzers depends on DLA class II genotype. Tissue Antigens 75, 712–719.

Woodard, J.C., 1982. Canine hypertrophic osteodystrophy, a study of the spontaneous disease in littermates. Veterinary Pathology 19, 337–354.

Wu, C., DeWan, A., Hoh, J., Wang, Z., 2011. A comparison of association methods correcting for population stratification in case-control studies. Annals of Human Genetics 75, 418–427.

Zanelli, E., Breedveld, F.C., de Vries, R.R., 2000. HLA association with autoimmune disease: A failure to protect? Rheumatology 39, 1060–1066.