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ORIGINAL ARTICLE

# DLA class II haplotypes show sex-specific associations with primary hypoadrenocorticism in Standard Poodle dogs

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## Abstract

Addison's disease (AD) is a life-threatening endocrine disorder that occurs spontaneously in both humans and dogs. Associations between MHC class II genes and AD have been shown in several human studies. Our goal was to identify MHC class II associations with AD in a large population of Standard Poodles, a breed highly predisposed to AD. We sequenced exon 2 of the class II genes DLA-DRB1, DLA-DQA1, and DLA-DQB1 in 110 affected and 101 unaffected Standard Poodles and tested for association with AD. After correcting for population structure, two haplotypes were found to confer risk of developing AD in a sex-specific manner: DLA-DRB1*015:01-DQA1*006:01-DQB1*023:01 in males ($x^2p = 0.03$, OR 2.1) and DLA-DRB1*009:01-DQA1*001:01-DQB1*008:01:1 in females ($x^2p = 0.02$, OR 8.43). Sex-specific associations have been previously described in human populations, but this is the first report of this kind in dogs. Consistent with findings in other studies, we found the DLA-DQA1*006:01 allele ($x^2p = 0.04$) to be associated with AD in males independent of haplotype. In females, the haplotype DLA-DRB1*009:01-DQA1*001:01-DQB1*008:01:1 confers a very high risk for developing AD, although its frequency was rare (9 of 124 females) in our study population. Further studies are warranted to validate the findings of this exploratory dataset and to assess the usefulness of this haplotype as a risk marker for AD in female Standard Poodles. Our results highlight the importance of evaluating MHC class II disease associations in large populations, and accounting for both biological sex and population structure.

**Keywords** Canine · Hypoadrenocorticism · Autoimmune disease · DLA · Major histocompatibility complex class II

## Introduction

Addison's disease (AD), also known as primary hypoadrenocorticism, is a chronic and life-threatening endocrinopathy caused by the immune-mediated destruction of the adrenal cortex that leaves affected individuals without certain hormones that are critical for normal homeostasis (Husebye and Løvås 2009; Mitchell and Pearce 2012; Pazderska et al. 2018). The disease is believed to be a complex genetic disorder in humans, with heritability estimates as high as 0.97 (Mitchell and Pearce 2012; Skov et al. 2017). Some genetic variants have been associated with AD; however, causative polymorphisms have not yet been identified (Mitchell and Pearce 2012; Pazderska et al. 2018; Falorni et al. 2008; Falorni et al. 2016). Several studies have shown a modest association between certain human leukocyte antigen (HLA) haplotypes and AD, in particular the MHC class II alleles HLA-DRB1*04:04 and HLA-DRB1*03:01 (Gombos et al. 2007; Skinningsrud et al. 2011; Erichsen et al. 2009; Betterle et al. 2013; Yu et al. 1999). These associations are consistent with the immune-mediated nature of AD, as HLA associations are common across many autoimmune disorders (Bilbao et al. 2003; Moutsianas and Gutierrez-Achury 2018; Bodis et al. 2018).

Dogs are the only species besides humans known to develop AD spontaneously (Greco 2007; Van Lanen and Sande 2014) and therefore provide a compelling model to study the disease in humans. In both species, the disease shares a similar

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presentation and clinical course (Greco 2007; Frank et al. 2013) and is believed to be mediated by both CD4 and CD8 lymphocytes (Bratland and Husebye 2011; Dawoodji et al. 2014; Freeman and Weetman 1992; Friedenberg et al. 2018; Rottembourg et al. 2010). Like humans, AD in dogs also occurs more frequently in certain sub-populations. In particular, AD is overrepresented in Standard Poodles, Portuguese Water Dogs, Cocker Spaniels, Nova Scotia Duck Tolling Retrievers (NSDTRs), and Bearded Collies (Burton et al. 1997; Chase, Sargan, Miller, Ostrander, & Lark; Famula et al. 2003; Hanson et al. 2016; A M Oberbauer et al. 2006; Anita M Oberbauer et al. 2002). In Standard Poodles, the prevalence of AD has been estimated to be 5–10% and the heritability as high as 0.8 (Famula et al. 2003).

The genetics of AD has been studied in several dog breeds; however, as in humans, a comprehensive understanding of canine AD genetics is lacking. One candidate gene study in Labrador Retrievers, Cocker Spaniels, and Springer Spaniels identified several single nucleotide polymorphisms (SNPs) in the genes *PTPN22* and *CTLA4* that are modestly associated with AD (Short et al. 2013). Another candidate gene study by the same investigators identified additional polymorphisms associated with AD in a broader set of dog breeds (Short et al. 2014). A genome-wide linkage study in Portuguese Water Dogs identified loci on chromosomes 12 and 37 linked with AD, but did not identify any specific causative variants (Chase et al. 2006). A genome-wide association study of Standard Poodles with AD did not identify any loci or SNPs significantly associated with disease, suggesting that the inheritance pattern in Standard Poodles may be polygenic (Friedenberg et al. 2017).

In addition to these genome-wide and candidate gene studies in dogs, four reports have examined the association of the class II dog leukocyte antigen (DLA) genes with AD (Massey et al. 2013; Pedersen et al. 2015; Hughes et al. 2010; Boag et al. 2015) (Supplemental Table 1). These studies identified both risk and protective haplotypes in dogs. For example, the haplotype DLA-DRB1*015:02-DQA*006:01-DQB*023:01 was found to confer risk in two breeds predisposed to AD, Standard Poodles and NSDTRs (Massey et al. 2013; Pedersen et al. 2015; Hughes et al. 2010). Additionally, the association of DLA-DQA1*006:01 with AD was a consistent finding in all four studies across several breeds (Supplemental Table 1).

Addison's disease is among the most significant health burdens in Standard Poodles (Pedersen et al. 2015). With the exception of the Pederson et al study, the number of affected Standard Poodles evaluated for DLA-AD associations has been small, potentially limiting the ability to draw definitive conclusions regarding associations between DLA haplotypes and AD in this highly predisposed breed. Therefore, additional studies including a large number of cases are warranted. The goal of this study was to identify MHC class II haplotypes associated with AD in a larger population of affected and unaffected Standard Poodles. Additionally, we sought to account for population structure within our dataset using genome-wide SNP data that had been simultaneously acquired for these dogs. We hypothesized that by using this larger dataset and accounting for population stratification, we would be able to definitively identify MHC class II haplotypes associated with AD in Standard Poodle dogs.

## Methods

### Study population and sample collection

We collected pedigrees and whole blood samples from 130 Standard Poodles with AD and 135 without AD. The requirements for a diagnosis of AD were minimum age of 1 year, serum sodium to potassium ratio < 27:1, ACTH stimulation test with pre- and post-ACTH cortisol levels ≤2 μg/dL, and a supportive clinical history (e.g., waxing/waning gastrointestinal upset, anorexia, weakness) (Van Lanen and Sande 2014). Unaffected dogs had to be at least 10 years of age and have either a baseline cortisol > 2 μg/dL or a normal ACTH stimulation test (Gold et al. 2016); we verified that unaffected dogs had not been receiving steroid-containing medications for at least 1 month prior to cortisol testing.

Blood was collected from each animal in EDTA tubes, and DNA was extracted using standard protocols of the DNeasy Blood and Tissue kit (Qiagen) or the Gentra Puregene kit (Qiagen).

### SNP Array genotyping

SNP genotypes from 180 of the 265 Standard Poodles evaluated in this study were derived from the Illumina 174K Canine HD BeadChip as described previously (Friedenberg et al. 2017). Approximately 0.4 μg of DNA from an additional 85 Standard Poodles was submitted to the Microarray Research Services Laboratory at ThermoFisher Scientific (Santa Clara, CA) for processing and genotyping using the 730K Axiom K9 HD Array. Genotyping and variant calling for these SNPs were carried out using Axiom Analysis Suite 3.1 using best practice guidelines provided by the manufacturer.

### Population outlier detection

SNPs from the higher-density Axiom array were pruned to include only those SNPs also present on the Illumina array and the two datasets were merged. SNP array-derived genotypes and samples were then filtered to exclude those dogs and SNPs meeting the following criteria: per-sample call rate < 0.9, inferred biological sex inconsistency based upon chromosome X heterozygosity < 0.05 for known males outside of the

pseudo-autosomal region, per-SNP call rate < 0.95, minor allele frequency < 0.02, or Mendelian genotype inconsistency based upon available pedigree data. Principal components were calculated, and sample outliers > 1.5× the interquartile range based upon the distance from the median centroid vector were detected and removed. Analyses were performed using PLINK 1.9 and R 3.4 (Purcell et al. 2007; R Core Team 2013).

### DLA genotyping

Exon 2 of DLA-DRB1, DLA-DQA1, and DLA-DQB1 were sequenced and genotyped according to the methods described elsewhere (Evans et al. 2017; Kennedy et al. 2006) with slight modifications. A T7 universal primer was added to the locus-specific forward primer for DLA-DQB1 and M13 universal forward and reverse primers were added to the locus-specific primers for DLA-DRB1. Complete primer sequences are provided in Supplemental Table 2. Reaction conditions were similar to those described in previous studies (Evans et al. 2017; Kennedy et al. 2006). Amplicons were sequenced at the University of Minnesota Genomics Center on an Applied Biosystems 3730xl DNA Analyzer. Alignment and variant calling were performed using Geneious 11.1 (Biomatters, Auckland, NZ). Consensus sequences at each locus were compared to a custom BLAST database of known class II DLA alleles in order to determine the DLA genotype for each dog. First, dogs that were homozygous at all three class II loci were selected. These dogs returned a 100% allele match based upon pairwise identity at each MHC locus and enabled the identification of various MHC haplotypes. Next, heterozygotes were compared to the BLAST database. If the BLAST search returned only two top hits, these hits were separated into haplotypes based upon the known homozygote haplotypes. If the BLAST search returned more than two top hits based upon pairwise identity, each of the top hits was manually inspected in order to determine which two were fully complementary at every heterozygous base; haplotypes were then called based upon the known homozygous haplotypes.

### Statistical analysis

Chi-square ($x^2$) tests were applied to class II haplotypes and individual MHC genes to detect association with AD, which is consistent with methods used in prior DLA association studies (Hughes et al. 2010; Perneger 1998; Rothman 1990; Massey et al. 2013; Shiel et al. 2014; Evans et al. 2015). Multiple testing correction was not employed as it can result in type II errors and consequently not recommended in exploratory datasets such as ours (Perneger 1998; Rothman 1990). The Cochran-Armitage trend test (CATT) was used to evaluate the association of haplotypes and genes with zygosity. Chi-square and CATT were also applied to the data subset by sex. A Woolf test for the homogeneity of odds ratios was used to evaluate whether the odds of developing AD was significantly different by sex. For all tests, $p \leq 0.05$ was considered significant. All statistical analyses were performed using R 3.4 (R Core Team 2013).

## Results

### Samples

Of the 265 Standard Poodles evaluated, four failed to meet SNP genotyping quality control. Principal components analysis based upon 127,199 SNPs across the remaining 261 dogs identified 18 population outliers. An additional 44 dogs were identified with known familial relationships within three generations based upon available pedigree information; 32 of these dogs were removed at random such that only one dog per known family was included. This yielded a final study cohort of 211 dogs.

Of the 211 dogs, 110 were affected and 101 were unaffected; 124 dogs were female (62 affected and 62 unaffected) and 87 were male (48 affected and 39 unaffected). At the time of sample collection, four dogs were intact (3 females, 1 male) and the remaining 207 dogs were spayed or neutered.

### DLA genotyping

DLA class II genotyping identified 16 haplotypes across all 211 dogs (Supplemental Table 3). Haplotype DLA-DRB1*015:01-DQA1*006:01-DQB1*023:01 (haplotype 1) was the most common, comprising 72% of the total haplotypes of the study population ($2n = 422$). Each of the next seven most frequent haplotypes comprised between 1 and 7% of the haplotypes. The remaining eight haplotypes had a count of 2 or less and were aggregated into the "other" haplotype category. These "other" haplotypes accounted for only 2% of the total haplotypes and were not evaluated statistically because of their low abundance. A summary of the haplotype frequencies in the entire population of 211 dogs and the distribution by sex is shown in Fig. 1. The distribution of the top eight haplotypes was not significantly different between males and females ($x^2p = 0.60$).

### Statistical analysis

**Study cohort (211 dogs)** Chi-square tests did not reveal a significant association between any MHC class II haplotype and AD (Table 1). Individual class II genes were also not associated with AD (Supplemental Table 4).

**Sex-based data subsets** Sex-specific risk haplotypes were identified for both males and females. In males, the most common haplotype, haplotype 1 (Table 1), was

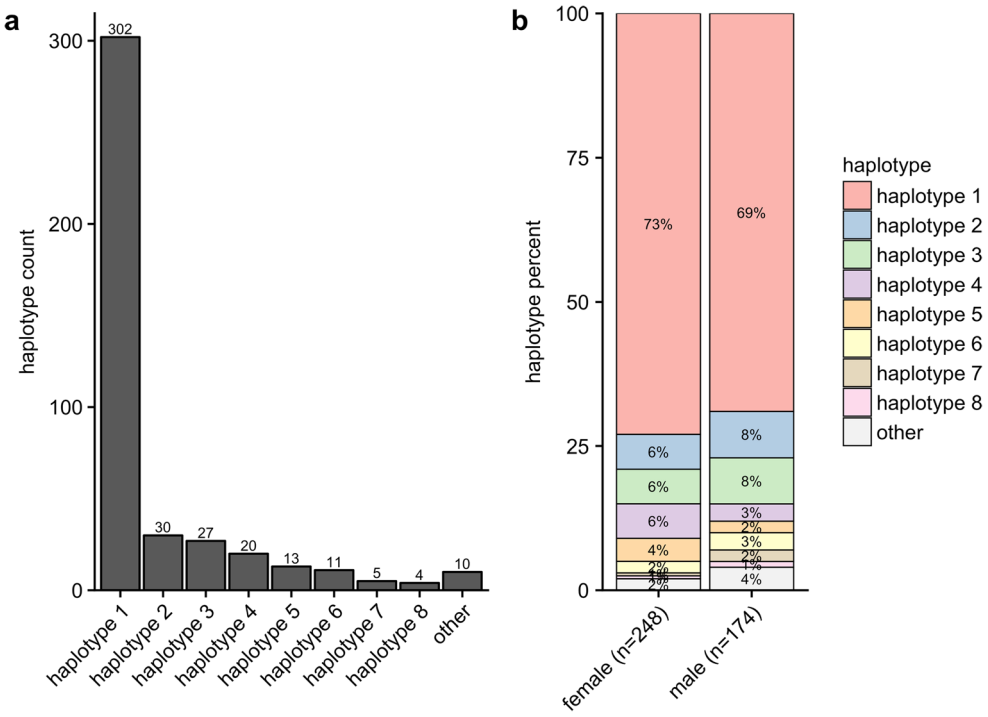


**Fig. 1** **a** Haplotype distribution in a study population of 211 Standard Poodles ($2n = 422$ haplotypes). Haplotypes with a frequency count of 2 or less were combined into one group labeled "other." Haplotype codes and corresponding identities are defined in Table 1. **b** Haplotype proportions segregated by sex. Haplotype counts by sex are displayed at the bottom of each stacked bar chart. Haplotype distributions were not found to be statistically different between males and females ($x^2p = 0.60$)

significantly associated with AD ($x^2p = 0.03$, Tables 2 and 3). Homozygosity of this haplotype in males also conferred an increased risk of AD (CATT $p = 0.03$, Tables 2 and 3). Association with individual DLA genes was also found in males: DLA-DQA1*006:01 ($x^2p = 0.03$, Supplemental Table 5) and DLA-DQB1* 023:01 ($x^2p = 0.04$, Supplemental Table 5). Homozygosity of these alleles increased the risk of disease (DLA-DQA1*006:01 CATT $p = 0.03$, DQB1*023:01 CATT $p = 0.04$). In females, DLA-DRB1*009:01-DQA1*001:01-DQB1*008:01:1, haplotype 5 (Table 1), was significantly associated with AD ($x^2p = 0.02$, Tables 2 and 3). However, the frequency of this haplotype was low (4% in females). All females with this haplotype were heterozygous, which precluded zygosity testing. DLA-DRB1*009:01 and DLA-DQB1*008:01:1 (both $x^2p = 0.02$) were each individually found to confer risk of disease in females.

**Table 1** Haplotype statistics—full dataset of 211 Standard Poodles (110 cases, 101 controls), $2n$ haplotypes

| Code | DLA haplotype | Addison's disease | | | Healthy controls | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *DRB1-DQA1-DQB1* | Hom (*n*) | Haplos (2*n*) | % | Hom (*n*) | Haplos (2*n*) | % | OR (95% CI) | $\chi^2$, *p* | CATT, *p* |
| 1 | 015:01–006:01–023:01 | 60 | 162 | 73.6 | 51 | 140 | 69.3 | 1.24 (0.79–1.93) | 0.33 | 0.20 |
| 2 | 015:01–009:01–001:01 | 4 | 18 | 8.2 | 1 | 12 | 5.9 | 1.41 (0.62–3.30) | 0.37 | 0.27 |
| 3 | 015:02–006:01–023:01 | 0 | 14 | 6.4 | 0 | 13 | 6.4 | 0.99 (0.42–2.35) | 0.98 | NA |
| 4 | 020:01–004:01–013:03 | 0 | 7 | 3.2 | 0 | 13 | 6.4 | 0.48 (0.16–1.32) | 0.12 | NA |
| 5 | 009:01–001:01–008:01:1 | 0 | 9 | 4.1 | 0 | 4 | 2.0 | 2.11 (0.58–9.52) | 0.21 | NA |
| 6 | 015:03–006:01–023:01 | 0 | 5 | 2.3 | 0 | 6 | 3.0 | 0.76 (0.18–3.04) | 0.65 | NA |
| 7 | 001:01–001:01–002:01 | 0 | 2 | 0.9 | 0 | 3 | 1.5 | 0.61 (0.05–5.38) | 0.59 | NA |
| 8 | 015:01–006:01–049:01 | 0 | 1 | 0.5 | 0 | 3 | 1.5 | 0.30 (0.01–3.81) | 0.28 | NA |
| NA | Other | 0 | 2 | 0.9 | 0 | 8 | 4.0 | NA | NA | NA |
| Totals | | 64 | 220 | 100% | 52 | 202 | 100% | | | |

A chi-square test was used to test for association between haplotypes and AD. A Cochran-Armitage Trend Test (CATT) was used to evaluate the relationship between zygosity and AD for each haplotype. Several haplotypes had insufficient numbers of homozygotes for CATT. The "other" category of haplotypes was not evaluated; these are denoted as "NA." The "Hom" column lists the number of dogs that are homozygous for each haplotype. The "Haplos" column is the total count of observations of a given haplotype. Percent (%) is the frequency of each haplotype by disease status

**Table 2** Haplotype statistics in males (87 total, 48 cases and 39 controls); 2*n* haplotypes

| | DLA haplotype | Addison's disease | | | Healthy controls | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Code | *DRB1-DQA1-DQB1* | Hom (*n*) | Haplos (2*n*) | % | Hom (*n*) | Haplos (2*n*) | % | OR (95% CI) | $\chi^2$, *p* | CATT, *p* |
| Males | | | | | | | | | | |
| 1 | 015:01–006:01–023:01 | 30 | 73 | 76.0 | 16 | 47 | 60.3 | 2.08 (1.04–4.24) | 0.03* | 0.03* |
| 2 | 015:01–009:01–001:01 | 2 | 7 | 7.3 | 0 | 7 | 9.0 | 0.80 (0.23–2.80) | 0.69 | 0.64 |
| 3 | 015:02–006:01–023:01 | 0 | 8 | 8.3 | 0 | 5 | 6.4 | 1.33 (0.36–5.38) | 0.63 | NA |
| 4 | 020:01–004:01–013:03 | 0 | 2 | 2.1 | 0 | 4 | 5.1 | 0.40 (0.04–2.85) | 0.27 | NA |
| 5 | 009:01–001:01–008:01:1 | 0 | 1 | 1.0 | 0 | 3 | 3.8 | 0.27 (0.01–3.38) | 0.22 | NA |
| 6 | 015:03–006:01–023:01 | 0 | 2 | 2.1 | 0 | 4 | 5.1 | 0.40 (0.04–2.85) | 0.27 | NA |
| 7 | 001:01–001:01–002:01 | 0 | 1 | 1.0 | 0 | 2 | 2.6 | 0.40 (0.01–7.86) | 0.44 | NA |
| NA | Other | 0 | 2 | 2.1 | 0 | 6 | 7.7 | NA | NA | NA |
| Totals | | 32 | 96 | 100% | 16 | 78 | 100% | | | |

A chi-square test was used to test for association between haplotypes and AD. A Cochran-Armitage Trend Test (CATT) was used to evaluate the relationship between zygosity and AD for each haplotype. *p* values ≤ 0.05 are indicated with an asterisk. Several haplotypes had insufficient numbers of homozygotes for CATT. The "other" category of haplotypes was not evaluated; these are denoted as "NA." The "Hom" column lists the number of dogs that are homozygous for each haplotype. The "Haplos" column is the total count of observations of a given haplotype. Percent (%) is the frequency of each haplotype by disease status

An overlap in the confidence intervals for the odds ratios in males and females was noted for both the male (haplotype 1) and female (haplotype 5) risk haplotypes; (1.04–4.24 [males] vs. 0.46–1.55 [females] for haplotype 1 and 0.01–3.38 [males] vs. 1.10–378.73 [females] for haplotype 5; Tables 2 and 3). A Woolf test for the homogeneity of odds ratios was used to test whether these confidence intervals were statistically different between the sexes despite sharing some overlap. Woolf *p*-values were significant for both risk haplotypes (haplotype 1 $p = 0.04$, haplotype 5 $p = 0.03$). This indicates that the confidence intervals in males and females are statistically different for both risk haplotypes and suggest that the observed sex bias of both risk haplotypes is significant.

We also evaluated whether each of these sex-specific haplotypes was associated with AD in our unfiltered population of 265 dogs (114 males, 151 females). Both haplotypes remained significant by sex (haplotype 1 $x^2p = 0.03$ in males, haplotype 5 $x^2p = 0.002$ in females) in the larger population of animals.

The identification of sex-based risk haplotypes suggested the possible influence of gonadal sex hormones on the development of AD. We therefore considered whether spay/neuter

**Table 3** Haplotype statistics in females (124 total, 62 cases and 62 controls); 2*n* haplotypes

| | DLA haplotype | Addison's disease | | | Healthy controls | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Code | *DRB1-DQA1-DQB1* | Hom (*n*) | Haplos (2*n*) | % | Hom (*n*) | Haplos (2*n*) | % | OR (95% CI) | $\chi^2$, *p* | CATT, *p* |
| Females | | | | | | | | | | |
| 1 | 015:01–006:01–023:01 | 30 | 89 | 71.8 | 35 | 93 | 75.0 | 0.85 (0.46–1.55) | 0.57 | 0.77 |
| 2 | 015:01–009:01–001:01 | 2 | 11 | 8.9 | 1 | 5 | 4.0 | 2.31 (0.71–8.75) | 0.12 | 0.13 |
| 3 | 015:02–006:01–023:01 | 0 | 6 | 4.8 | 0 | 8 | 6.5 | 0.74 (0.20–2.51) | 0.58 | NA |
| 4 | 020:01–004:01–013:03 | 0 | 5 | 4.0 | 0 | 9 | 7.3 | 0.54 (0.14–1.85) | 0.27 | NA |
| 5 | 009:01–001:01–008:01:1 | 0 | 8 | 6.5 | 0 | 1 | 0.8 | 8.43 (1.10–378.73) | 0.02* | NA |
| 6 | 015:03–006:01–023:01 | 0 | 3 | 2.4 | 0 | 2 | 1.6 | 1.51 (0.17–18.37) | 0.65 | NA |
| NA | Other | 0 | 2 | 1.6 | 0 | 6 | 4.8 | NA | NA | NA |
| Totals | | 32 | 124 | 100% | 36 | 124 | 100% | | | |

A chi-square test was used to test for association between haplotypes and AD. A Cochran-Armitage Trend Test (CATT) was used to evaluate the relationship between zygosity and AD for each haplotype. *p* values ≤ 0.05 are indicated with an asterisk. Several haplotypes had insufficient numbers of homozygotes for CATT. The "other" category of haplotypes was not evaluated; these are denoted as "NA." The "Hom" column lists the number of dogs that are homozygous for each haplotype. The "Haplos" column is the total count of observations of a given haplotype. Percent (%) is the frequency of each haplotype by disease status

status might influence disease status. For both sexes, however, too few animals were intact (3 females, 1 male) to perform this analysis.

## Discussion

In this large study evaluating the association of MHC class II haplotypes with AD in Standard Poodles, we found two class II haplotypes associated with the disease in a sex-specific manner: haplotype 1 in males and haplotype 5 in females. Sex-specific associations have been previously described in human populations (Cavan et al. 1994; McCombe et al. 2006; Hughes et al. 2012), but this is the first report of this kind in dogs. In contrast to a previous study in Standard Poodles (Massey et al. 2013; Pedersen et al. 2015), we did not find any MHC haplotypes significantly associated with AD when we evaluated our data in a sex-agnostic manner.

Evidence for sex-based associations between class II MHC genes and autoimmune diseases has been well-described in human studies but has not been previously described in canine study populations (Irizar et al. 2012; Schneider-Hohendorf et al. 2018; Taneja et al. 2007; Behrens et al. 2010; Celius et al. 2000; Duquette et al. 1992; Czaja and Donaldson 2002). We therefore sought to examine if sex-based associations existed in our population of Standard Poodles with AD. When evaluated by sex, haplotype 1 was identified as a risk haplotype in males ($x^2p = 0.03$). This haplotype has not been previously associated with AD in Standard Poodles; however, it has been described as a risk haplotype for AD in both English Cocker Spaniels and English Springer Spaniels (Massey et al. 2013). We also found that in males, the association trend increases with zygosity (CATT $p = 0.03$). This finding is consistent with the co-dominant expression of MHC molecules (Murphy and Weaver 2017) and lends additional support that this haplotype confers risk for AD in male Standard Poodle dogs.

We also found associations with specific alleles of individual MHC class II genes in males, most notably DLA-DQA1*006:01 ($x^2p = 0.04$) and DLA-DQB1*023:01 ($x^2p = 0.03$). These findings are consistent with several prior studies. For example, DLA-DQA1*006:01 was significantly associated with seropositive P450 side-chain cleavage enzyme autoantibody status in dogs with AD across multiple breeds (Boag et al. 2015). In separate studies of Nova Scotia Duck Tolling Retrievers (Hughes et al. 2010) and Standard Poodles (Massey et al. 2013), the authors found an association between AD and haplotype 3. While our findings differ at the DLA-DRB1 locus, they are consistent at both DQ loci. The DLA-DQA1 locus in particular has been associated with AD across all of these studies, suggesting that DLA-DQA1*006:01 may be a key driver of AD across multiple dog breeds. Future research is warranted to explore the impact of this allele more broadly.

Also intriguing is the risk haplotype we identified in females, haplotype 5 ($x^2p = 0.02$, OR 8.43): 8 of 9 females we identified with this haplotype are affected (Tables 2 and 3). This haplotype has been previously reported as a risk haplotype in other breeds, including Bearded Collies and English Cocker Spaniels (Massey et al. 2013), but our finding in Standard Poodles is novel. Interestingly, all of the females we identified with this haplotype were heterozygous, suggesting that haplotype 5 confers a high risk of AD. Given its low frequency as well as the absence of homozygotes, it is possible that Standard Poodle breeders have bred away from dogs with this haplotype, or that it confers some type of genetic disadvantage. Regardless, follow-up validation studies are needed as work by Safra et al. has demonstrated the possibility of spurious associations of haplotypes occurring in low frequencies (Safra et al. 2011). Validation work would require a significantly larger sample size in order to enrich for this low-frequency haplotype. Other validation approaches such as large linkage studies or SNP genotyping across the entire DLA region could also be considered (Safra et al. 2011).

While our findings are consistent with many prior DLA association studies on AD, the sex specificity of our associations and the new risk haplotype we identified in Standard Poodles are novel. There are several possible explanations for the differences between our findings and prior work in Standard Poodles. To the best of our knowledge, for example, earlier studies have not evaluated their datasets in a sex-specific manner (Massey et al. 2013; Hughes et al. 2010; Pedersen et al. 2015). Additionally, our study included the largest number of affected Standard Poodles (101) to date (Pedersen et al. 2015; Massey et al. 2013; Boag et al. 2015). Our dataset was also filtered to remove population outliers based upon SNP data and excluded dogs with known genetic relatedness based upon pedigree information. All of these steps are likely to have increased our ability to detect novel associations between MHC class II alleles and AD in this exploratory dataset, while minimizing the impact of population stratification and inbreeding on our findings.

At the present time, a sex-specific association for MHC class II haplotypes and AD has not been reported in human studies. However, sex-based associations of MHC class II and other autoimmune diseases have been reported in human studies and include Guillain-Barré syndrome, chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), Graves' disease, rheumatoid arthritis (including murine models), multiple sclerosis, and autoimmune hepatitis (Behrens et al. 2010; Celius et al. 2000; Czaja and Donaldson 2002; Duquette et al. 1992; Irizar et al. 2012;

Schneider-Hohendorf et al. 2018; Taneja et al. 2007). The biological underpinnings for sex-specific risk haplotypes are at present unknown and have only recently started to be explored by researchers. The sex-differentiated results of our study suggest that exposures to male and female sex hormones may be influential in the development of canine AD. Consider, for example, a recent study in humans by Schneider-Hohendorf et al. that demonstrated significant differences in T cell: MHC interactions between males and females, leading the authors to conclude that HLA association studies should be evaluated with regard to sex (Schneider-Hohendorf et al. 2018). Specifically, biological sex was shown to be associated with T cell receptor beta chain variable segment usage as well as dynamics of T cell expansion among study participants with the same MHC background. Given that the T cell receptor repertoire develops largely prenatally (Haynes and Heinly 1995), as well as the strong influence of the MHC on the development of the T cell repertoire (Krovi and Gapin 2016; Jerne 1971), sex hormones may exert an effect prior to spaying or neutering. This is particularly noteworthy given that the vast majority of dogs in our study population were spayed or neutered at the time of diagnosis, but were likely intact during thymic T cell development both in utero and in the first several months of life.

Another explanation for sex-specific MHC class II associations with autoimmune disease is the transcriptional regulation of autoimmune regulator (AIRE) by sex hormones in the thymus during T cell development. Human and murine studies have revealed that the AIRE enzyme is upregulated in males and downregulated in females (Dragin et al. 2016; Zhu et al. 2016). AIRE controls the repertoire of self-peptides which MHC molecules are exposed to during T cell development (Perniola 2018). In theory, the same MHC haplotype can be exposed to different repertoires of self-peptides on the basis of sex alone. Additional explanations for the sex bias of autoimmune disease may ultimately play a role in influencing sex-based MHC associations. These include gene dosage effects from X-chromosomes, sex-based epigenetic patterning, and sex-specific environmental exposures (Dai and Ahmed 2014; Gabory et al. 2009; Ortona et al. 2016; Liu et al. 2015). As future studies in both humans and dogs identify additional sex-specific associations between the MHC and various autoimmune diseases, the mechanisms causing these sex differences may become increasingly apparent.

In summary, this large study of MHC class II associations with AD identified two novel risk haplotypes for Standard Poodles which associated in a sex-specific manner: haplotype 1 in males and haplotype 5 in females. These novel findings were facilitated by a robust approach to study design which included enrolling a large number of cases and an analysis which accounted for both sex and population structure. Follow-up studies are warranted to determine the clinical utility of these associations as genetic markers of risk for AD and to further validate the findings of this exploratory dataset. Functional studies are also needed to gain insight into the molecular basis by which these associations contribute to the pathogenesis of AD.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** Blood samples were collected at the University of Minnesota and North Carolina State University. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

**Informed consent** Signed consent forms, approved by each institution's Institutional Animal Care and Use Committee, were collected from the owners of each study participant as part of the enrollment process.

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