# Genome-wide association analysis in West Highland White Terriers with atopic dermatitis

Cary S. Agler[a], Steven Friedenberg[b,1], Thierry Olivry[a,c], Kate M. Meurs[a,c], Natasha J. Olby[a,c,*]

[a] Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, NC, 27607, USA
[b] Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of Minnesota, Saint Paul, MN, 55108, USA
[c] Comparative Medicine Institute, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, NC, 27607, USA

## ARTICLE INFO

## ABSTRACT

*Background:* Atopic dermatitis (AD) is a common disease of dogs and humans. In both species, the interplay of genetic and environmental factors affect disease expression. In dogs with AD, differences in the breed studied and in their geographical origin have led to heterogeneity in genetic association and while different loci have been identified, a causative genetic mutation has not. We hypothesized that AD could be mapped in a large cohort of rigorously phenotyped, geographically restricted West Highland White Terriers (WHWT), a breed with a high prevalence of the disease.
*Objectives:* A) Collect phenotypes and DNA from a large cohort of WHWT born in the USA. B) Perform a genome-wide association study (GWAS) for AD in these dogs to identify associated regions and genes of interest. C) Sequence genes of interest to identify pathologic variants.
*Methods:* We collected DNA from 96 WHWT with AD and 87 controls from the same breed. DNA was isolated and dogs were genotyped using the Illumina CanineHD BeadChip. A GWAS was performed using EMMAX and associated regions were examined for genes of interest. Genes with possible relevance to AD were examined more closely in two affected and two normal WHWT using next-generation sequencing. Variants in these genes that were unique to the two affected WHWT were compared to a database of variants derived from whole genome sequencing of 200 non-WHWT dogs across 33 additional breeds.
*Results:* The GWAS identified a 2.7 Mb genomic region on CFA3 that included 37 genes. There was a missense variant in the *F2R* gene in both affected dogs but this variant was also found in 35 dogs in 9 breeds in the database of whole genome sequences for whom the phenotype regarding atopic dermatitis was unknown.
*Conclusions:* Atopic dermatitis in WHWT is associated with a region on CFA3 that contains several candidate genes. Of these, a homozygous variant in the *F2R* gene present in multiple breeds that also suffer from AD warrants further evaluation.

## 1. Introduction

Canine atopic dermatitis (AD) is a common, chronically recurrent, pruritic allergic skin disease with characteristic skin lesions that include erythema, excoriations, lichenification and self-induced alopecia at specific body locations (face, feet, axillae, groin, abdomen, perineum and flexural areas of the limbs) (Griffin and DeBoer, 2001; Favrot et al., 2010; Hensel et al., 2015). The pathogenesis of this disease is complex and it involves, at least, allergen-specific IgE, mononuclear and eosinophilic skin inflammation, pro-inflammatory, pruritogenic and T-helper (Th)- 2 and Th-22 cytokines, and a secondary epidermal barrier dysfunction predisposing the skin to bacterial and yeast dysbiosis and infections (Marsella et al., 2012; Olivry et al., 2016). After the landmark discovery, in 2006, that, in some human patients, AD was linked to null mutations of *FLG* encoding the upper epidermal protein filaggrin (Palmer et al., 2006), the development of modern molecular biology techniques has helped establish that human AD is a genetically heterogeneous syndrome associated with predisposing mutations, not only on *FLG*, but also on other genes encoding proteins involved in the establishment of a functional epidermal barrier or genes of proteins involved in the innate and adaptive immune systems (reviewed in Mu et al., 2014; Bin and Leung, 2016).

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* Corresponding author at: Department of Clinical Sciences, College of Veterinary Medicine, 1060 William Moore Drive, NC State University, Raleigh, NC, 27606, USA.

*E-mail address:* njolby@ncsu.edu (N.J. Olby).

[1] Joint first authors.

Certain dog breeds are known for their high prevalence for AD, for example terrier breeds including West Highland white terriers (WHWT) (Jaeger et al., 2010). In Golden and Labrador Retrievers, nearly half of the risk for the disease can be attributed to genetic variation (Shaw et al., 2004). Previous research into the genetics of canine AD in WHWT suggests that it is influenced by more than one gene, or by a gene with low penetrance (Salzmann et al., 2011).

A few genome-wide association studies (GWAS) of AD have been performed in several dog breeds including the WHWT (reviewed in Nuttall, 2013; Bizikova et al., 2015). The first GWAS used dogs from different breeds including Boxers, German shepherd dogs, Labrador and golden retrievers, Shiba inu, shih tzu, pit bulls, and WHWT identified three SNPs associated with AD in all eight breeds studied (Wood et al., 2009a, b). In addition, the authors reported four SNPs associated with AD in 18 WHWT cases and 48 WHWT controls (Wood et al., 2009a, b). The same group later evaluated SNPs in AD candidate genes in golden retrievers and seven other dog breeds including the WHWT (Wood et al., 2010). A SNP was identified in the *thymic stromal lymphopoietin receptor* (*TSLPR*) gene that was associated with AD in all eight breeds (Roque et al., 2011), while another SNP in *FLG* was associated with AD in Labrador retrievers in the United Kingdom (Wood et al., 2010). Whereas all studies corrected for multiple testing, only two of these accounted for population stratification. While the assignment to the affected/normal phenotype status was relatively similar in all studies, with a diagnosis made by veterinary dermatologists based on compatible history and clinical signs and exclusion of other causes for pruritus, only Roque *el al.* described criteria used to assign a normal phenotype (i.e. dogs greater than 5 years of age from a non-atopic family; Roque et al., 2012). In addition, all the WHWT studies used different cohorts of dogs from various geographical regions and Wood and colleagues reported different genetic associations in dogs of the same breed but living in different geographic areas (Wood et al., 2009a,b). As a support for restricting the population of dogs tested to ensure subject homogeneity, the most recent GWAS tested dogs from a single breed and a single country, and this led to the identification of some positive associations. For example, the analysis of samples from 35 atopic and 25 healthy WHWT in Australia identified significantly associated SNPs in a 1.3 Mb locus on CFA17 (Roque et al., 2012). More recently, a study in atopic German shepherd dogs from Sweden associated the trait to a 1.5 Mb region of CFA27 (Tengvall et al., 2013). Altogether, these GWAS results clearly establish that canine AD is a genetically-heterogeneous syndrome and that future studies should be restricted to individuals from a single breed and geographical area (Nuttall, 2013).

We herein report results of a GWAS performed in a cohort of WHWT from the USA, using the Illumina's CanineHD SNP set with over 170,000 SNPs, with the aim of ultimately identifying gene(s) associated with AD in this canine breed long known to be predisposed to develop AD in this as in other countries.

## 2. Material and methods

### 2.1. Sample collection

West Highland white terriers were recruited through advertisement on the breed society webpage (http://www.westieclubamerica.com/), by referral from veterinary dermatologists across the USA and from clients being seen in the dermatology clinic at NC State University's Veterinary Hospital. We also used other DNA samples collected from WHWTs and included in a previous linkage study (Salzmann et al., 2011). These were selected based on adequate quantities of high quality DNA and clinical phenotype. The diagnosis of AD was made according to standard criteria (Favrot et al., 2010; Olivry, 2010) by one of the authors (TO). In brief, affected dogs generally exhibited a juvenile onset of pruritic erythematous skin lesions with a characteristic distribution, intradermal testing or allergen-specific IgE serology results showing hypersensitivity against environmental allergens such as house dust

mites, molds, or pollens, and a positive response of skin lesions to anti-inflammatory drugs known to be effective for treatment of canine AD (e.g. oral or topical glucocorticoids or calcineurin inhibitors). Included WHWTs could have environmental and/food-induced AD (Olivry et al., 2007). Furthermore, resembling pruritic skin diseases such as other manifestations of food allergies, scabies, and skin infections were excluded. All relevant clinical data were extracted from an *ad hoc* questionnaire, along with the review of results of any additional tests performed. The health questionnaires enquired about each dog's age, pedigree, allergies (including specific questions about the diagnosis and treatment of skin lesions), as well as any family history of allergic disease (if applicable). Multigenerational pedigrees were provided by the owners, when available. West Highland white terriers of at least five years of age with no signs of AD and without a family history of AD or resembling pruritic skin lesions were considered normal and served as controls. We extracted DNA from whole blood using QIAamp DNA Blood Midi Kit (Qiagen; Valencia, CA) or from saliva using Oragene Animal kit (DNA Genotek; Kanata, Ontario). The DNA concentrations were measured using a ND-1000 UV–vis NanoDrop spectrophotometer (Thermo Scientific; Wilmington, DE). All protocols were performed after approval by the NC State University's Institutional Animal Care and Use Committee.

### 2.2. Genotyping and data analysis

Ninety-seven (53 male and 44 female) cases and 89 (36 male and 53 female) control dogs were genotyped using Illumina's CanineHD (173,662 SNPs) genotyping BeadChip (Illumina; San Diego, CA). The assays were performed at GeneSeek (Lincoln, NE) according to the manufacturer's instructions and the raw data was returned as IDAT files that were uploaded into Illumina's Genome Studio software (Illumina; San Diego, CA) to be analyzed. Data were pruned using the PLINK toolset v1.07 (http://pngu.mgh.harvard.edu/-purcell/plink/) (Purcell et al., 2007) so that dogs with less than a 95% call rate, SNPs having a minor allele frequency of less than 1%, and missing genotype calls greater than 10% were removed from further analysis. We checked SNP results on the X and Y chromosomes to confirm gender status and as a quality control measure. The pruned dataset was then used to perform case-control association analysis using the program EMMAX (Kang et al., 2010) (http://genetics.cs.ucla.edu/emmax/install.html) to control for any confounding factors such as population stratification, family structure, or cryptic relatedness. JMP Genomics (SAS; Cary, NC) was used to create a quantile-quantile (Q-Q) plot to determine whether the observed distribution of the test statistics followed the expected distribution and whether bias was present in the data. The CanFam3 reference sequence (http://genome.ucsc.edu) was used to investigate genes in the region(s) of the genome in which the most highly associated SNPs were detected by the GWAS.

### 2.3. Whole genome sequencing

Approximately 3 μg of DNA from two affected and two normal WHWT was submitted for library preparation and whole genome sequencing at The University of Missouri DNA Core. Each dog was sequenced using a 100 bp paired-end read configuration in a single lane of an Illumina HiSeq 2000 high-throughput sequencing system. These reads have been made publicly available at NCBI's Short Read Archive at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA476342. Variant calling from WGS data was performed using a standardized bioinformatics pipeline for all samples as described previously. (Friedenberg and Meurs, 2016) Briefly, sequence reads were trimmed using Trimmomatic 0.32 (Bolger et al., 2014) to a minimum phred-scaled base quality score of 30 at the start and end of each read with a minimum read length of 70 bp, and aligned to the CanFam3 reference sequence (Lindblad-Toh et al., 2005) using BWA 0.7.10 (Li and Durbin, 2009). Reference aligned reads were prepared for analysis using Picard Tools

2.5 (http://broadinstitute.github.io/picard) and GATK 3.4 (McKenna et al., 2010) following best practices for base quality score recalibration and indel realignment (Broad Institute, Cambridge, MA)(DePristo et al., 2011; Van der Auwera et al., 2013). Variant calls were made using GATK's HaplotypeCaller, and variant quality score recalibration (VQSR) was performed using sites from dbSNP 146 and the Illumina 174 K CanineHD BeadChip as training resources. We applied a VQSR tranche sensitivity cutoff of 99.9% to SNPs and 99% to indels for use in downstream analyses; genotype calls with a phred-scaled quality score < 20 were flagged but not removed from the variant call-set. Variant effects were annotated using SnpEff 4.1 (Cingolani et al., 2012).

Heterozygous or homozygous variants common to both affected dogs within a region of interest on chromosome 3 (CanFam3 coordinates 29,536,260-32,327,442) were compared to those in the normal dogs. The resulting dataset was subsequently filtered against a population of 200 non-WHWT dogs across 33 different breeds whose entire genomes were sequenced as part of ongoing work in our laboratory. These breeds included: 22 boxers, 20 standard poodles, 19 great Danes, 13 Yorkshire terriers, 13 Cavalier King Charles spaniels, 11 miniature schnauzers,10 dachshunds, 10 miniature poodles, and 82 dogs of 25 additional breeds. Whole genome sequences from these dogs were processed using a similar bioinformatics pipeline as described above. Variants were evaluated based on the severity of the predicted effect. Variants we considered most impactful included frameshifts, inframe insertions and deletions, premature-start, stop-gained, missense, and splice region changes.

## 3. Results

DNA samples from a total of 186 (97 cases, 86 controls) WHWT were collected and genotyped on 173,662 SNPs using the Illumina CanineHD BeadChip. The cases' ages ranged from 1 to 15 years old (mean ± SD: 6.1 ± 3.4 years) while the controls' age range was from 5 to 16 years old (mean ± SD: 8.7 ± 2.4 years). Twenty-seven of the cases and 44 of the controls had been collected for the linkage study described previously (Salzmann et al., 2011). Three dogs were removed during data pruning; one control dog was removed because it had a genotyping rate below 95% and one case and one control were removed due to incorrect sex status which likely occurred during sample acquisition and not during genotyping. In summary, the association analysis was performed using 96 (52 male and 44 female) cases and 87 (34 male and 53 female) controls with a total data set consisting of 117,576 SNPs after pruning. The case-control association study performed in EMMAX controlled for population biases effectively as evidenced in the Q-Q plot (Fig. 1). We did not identify any SNPs that were significantly associated with phenotype following Bonferroni correction. However, if we established significance at $P < 10^{-5}$ (Moskvina and Schmidt, 2008), six SNPs, five of them on CFA3, met the criteria for significance (Fig. 2). An MDS plot is provided in the supplementary data (supp data 1).

The genomic regions containing SNPs with the lowest P-values were inspected for presence of genes of interest. Eight of the top 12 associated SNPs were present in a 2.7 Mb region on chromosome 3 (Fig. 3). This 2.7 Mb genomic region contained approximately 37 genes such as *AGGF1, CRHBP, IQGAP2, F2R, F2RL1, F2RL2, MRPL42, SAP18*, and *AZNI1* in addition to seven olfactory receptor genes. The SNPs with the smallest P-values were located in a region containing a single gene: *TUBGCP5*; tubulin gamma complex associated protein 5. The other cluster of three SNPs were located between 30,593,968-31,021,430 bp and contained the genes *PSPH, POC5, EIF3F, ANKDD1B, POLK, HMGCR*, and *COL4A3BP*. Of these genes, *HMGCR* (encoding HMG-CoA reductase) was considered a potential candidate gene because of its importance in regulating cholesterol synthesis in the epidermis (Menon et al., 1992; Proksch et al., 1993). *COL4A3BP,* a ceramide transporter, was also identified as a candidate gene due to its role in generating stratum corneum intracellular lipids, critical for prevention of water
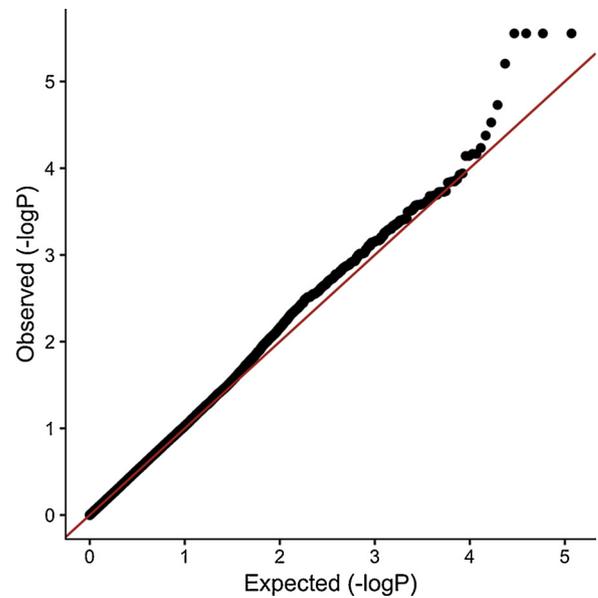


**Fig. 1.** Q-Q plot of the P-values obtained performing a GWAS using EMMAX.

loss. Finally *F2RL1* (also known as PAR-2) *and F2R (PAR-1)* were both considered genes of interest due to their involvement in inflammation after activation by endogenous and exogenous (e.g. allergenic) proteases such as those involved in desquamation or coming from environmental allergens.

Whole genome sequencing of the two affected and two normal dogs described above resulted in an average genome-wide depth of coverage of 24–32×. Within the 2.8 Mb region of interest and across all four WHWT, we detected 9308 variants (bialleleic and multiallelic). These variants had 13,572 distinct variant effects contained within 43 annotated genes. Among the variant effects, two were predicted by Ensembl's Variant Effect Predictor (https://useast.ensembl.org/info/docs/tools/vep/index.html) (VEP) to have a high impact on gene function, 32 a moderate impact, 42 a low impact, and 13,496 a modifier impact. Examination of the region on CFA3 identified a missense variant predicted to have a moderate effect on protein function or expression in *F2R* at 29,873,579 (G to A) that was homozygous in both affected WHWT but heterozygous in the normal WHWT. No other missense variants whose genotype was common to both affected WHWT and different from the genotypes in both normal WHWT were identified in this region. However, further examination of all dogs in our database of whole genome sequences confirmed presence of this *F2R* variant in multiple dog breeds (standard, (n = 12, 3 dogs homozygous), miniature, (n = 2, 1 dog homozygous), and toy poodle (n = 1), Cairn terrier (n = 1), Yorkshire terrier, (n = 12, 7 dogs homozygous), Irish setter, (n = 2, 2 dogs homozygous), great Dane (n = 3), labradoodle (n = 1), and Boykin terrier (n = 1, homozygous). The AD phenotype of these non-WHWT breed dogs in our database of whole genome sequences was unknown. Further evaluation of this variant across multiple breeds with known phenotype is warranted.

## 4. Discussion

This GWAS using 96 WHWT cases and 87 WHWT controls identified six SNPs that were significantly associated with AD using a significance level of $P < 10^{-5}$. Two regions on CFA3 contained multiple SNPs with the lowest P-values. These regions were inspected for genes of interest and one gene, *F2R*, contained a missense variant that segregated with the phenotype. This variant was found in other breeds in our database, but their AD phenotype was unknown. We could not confirm the associations identified in other studies of AD in WHWT or other breeds.

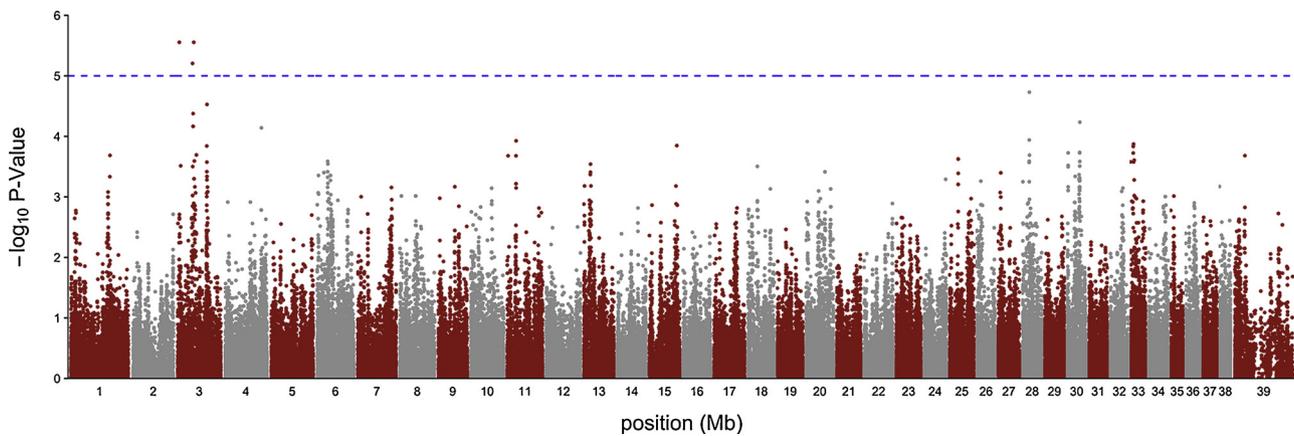This GWAS was performed in a large cohort of WHWT in which

**Fig. 2.** Manhattan skyline plot of the results of a GWAS performed using EMMAX. Unadjusted $-\log_{10}$ p-values are plotted against chromosome using 96 WHWT cases, 87 WHWT controls, and 117,576 SNPs. The dashed horizontal line indicates $p = 10^{-5}$. The genomic inflation factor was 1.04.

stringent criteria were used to establish both affected and normal status. Bias within the population was controlled for effectively using EMMAX and we identified a new locus on CFA3. This region has not been described in previous genetic studies of canine AD (Wood et al., Roque et al., Tengvall et al.). The SNPs in the locus were clustered in two smaller regions. The first region spanning 32,281,999 - 32,327,442 bp contains the gene *TUBGCP5*; tubulin gamma complex associated protein 5. This protein is a subunit of the gamma tubulin complex which localizes to the centrosome and associates with microtubules (Murphy et al., 2001). Copy number variation involving this gene in humans has been associated with Prader-Willi syndrome (Bittel et al., 2006), Alzheimer's disease (Ghani et al., 2012), and schizophrenia (Stefansson et al., 2008; Zhao et al., 2012); however, none of these diseases are associated with skin lesions. The second region spanning 30,593,968 - 31,021,430 bp contains genes such as *PSPH, POC5, EIF3F, ANKDD1B, POLK, HMGCR*, and *COL4A3BP*. Among those, *HMGCR* is the most likely candidate for AD. Indeed, HMGCR, a 3-hydroxy-3-methylglutaryl-CoA reductase, is the rate-limiting enzyme for cholesterol synthesis (Rodwell et al., 1976). The presence of cholesterol in the epidermis is important in maintaining the skin's permeability barrier

(Feingold et al., 1990) which is disrupted in AD (Menon et al., 1992; Proksch et al., 1993; Marsella et al., 2012). However, sequencing of this gene in two affected and two normal dogs did not reveal variants that segregated with the phenotype. The *COL4A3BP*, also known as *CERT* is known to encode a ceramide transporter and this function was considered relevant for AD due to the importance of stratum corneum ceramides in ensuring a proper epidermal barrier function. Regrettably, here again, we did not find allelic differences between the two affected and two normal WHWTs. The extended 2.7 Mb genomic region from 29,536,260 - 32,327,442 bp contains the genes *F2R* (thrombin receptor, PAR-1), *F2RL1* (thrombin receptor-like 1, protease activated receptor 2, PAR-2), and *F2RL2* (thrombin receptor-like 2, PAR-3). These are members of the protease-activated receptor (PAR) family. Interestingly *F2RL1* (PAR-2), is expressed in lesional skin of AD and is involved in homeostasis of the skin's permeability barrier (Maeda et al., 2009; Lee et al., 2010). While we identified a missense mutation in *F2R* that segregated between two normal WHWTs that were heterozygotes and two affected dogs that were homozygotes for this change, the presence of this variant in multiple breeds in our pipeline confirmed that this is a common canine variant. It is also possible that this variant is not
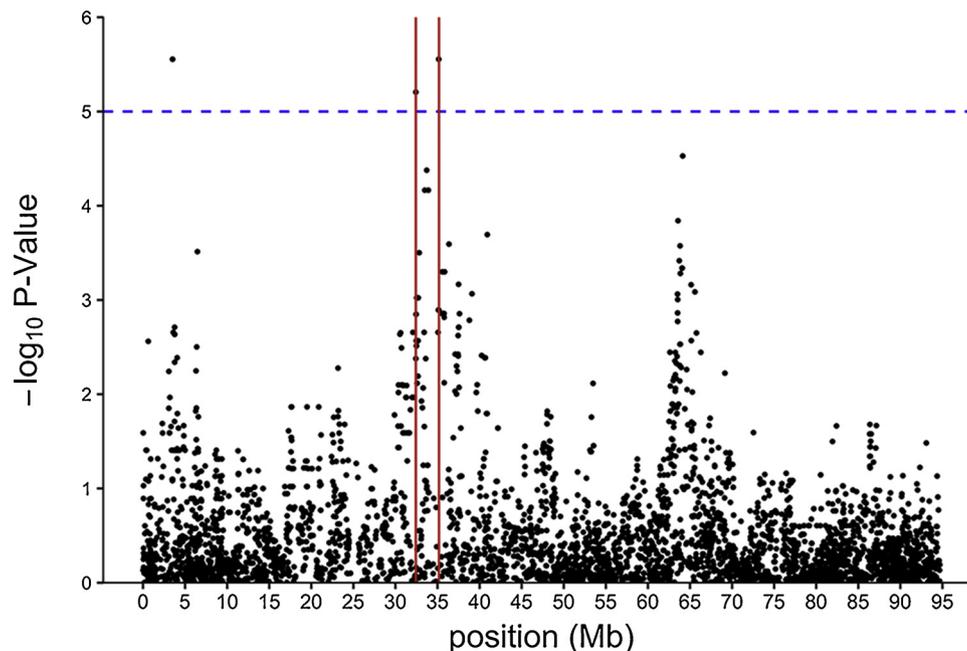


**Fig. 3.** The results of the same GWAS in greater detail on CFA3. The dashed horizontal line indicates $p = 10^{-5}$ and the vertical lines outline the 2.7MB region containing genes of particular interest.

expressed in transcripts of the gene specific to the skin. The lack of phenotypic information on atopic dermatitis in the dogs in our pipeline meant that further interpretation of the finding was not possible. Additional exploration of the importance of this F2R variant would include Sanger sequencing of the variant in a population of well-phenotyped WHWT and in other breeds of dog with and without confirmed atopic dermatitis. It is also important to note that our approach of identifying variants that were homozygous or heterozygous in cases but not controls would identify variants associated with a simple dominant or recessive trait but would not identify variants associated with a more complex genetic disease.

There are several different studies exploring the genetic basis of canine AD (Wood et al., 2009a,b, Wood et al., 2010; Roque et al., 2011; Suriyaphol et al., 2011; Roque et al., 2012; Tengvall et al., 2013). Of these, a study using 35 WHWT cases and 25 WHWT controls in Australia identified significantly associated SNPs in a 1.3 Mb locus on CFA17. Further evaluation of this locus suggested that polymorphisms in the candidate gene *PTPN22* in this locus may play a role in atopic dermatitis (Roque et al., 2011, 2012b). Another group performed a GWAS using multiple breeds of dog including 18 affected and 48 normal WHWT from the USA and UK and identified a SNP in *TSLPR* on CFAX as significantly associated with the trait across all breeds examined (Wood et al., 2009a,b, 2010). Finally, another study in German Shepherd dogs identified a locus on CFA27 (Tengvall et al., 2013). None of the loci identified in these studies coincided with those found in our GWAS using 96 affected and 87 normal WHWTs. The previous studies used slightly different genotyping methods including: the SNP20 Illumina BeadChip (Wood et al., 2009a,b), selected SNPs in candidate genes (Wood et al., 2010), and the Affymetrix Platinum Panel dog array (Roque et al., 2012). The CanineHD SNP set used in our research contained 14 of the 22 SNPs reported previously. All studies corrected for multiple testing however only two of the studies account for population stratification (Roque et al., 2012; Wood et al., 2010). While affected phenotype assignment was relatively similar in all studies with diagnosis by veterinary dermatologists based on compatible history and clinical signs and exclusion of other causes for pruritus, only Roque el al. described criteria used to assign normal phenotype that included dogs greater than 5 years of age from a non-atopic family. In addition, all the WHWT studies used different cohorts of dogs from different geographical regions and Wood et al. reported different associations in dogs of the same breed from different geographic regions (Wood et al., 2010). This study used a WHWT population that came from the US; whereas, the other three studies used WHWT populations from the UK and Australia, which could explain the difference in the associations detected. The filaggrin gene, mutations of which have been identified in human AD, is located on chromosome 17 and has not had associations identified in WHWT; however, associations with atopic dermatitis in British Labrador retrievers and a small group of poodles, shih tzus and pugs have been reported (Wood et al., 2010; Suriyaphol et al., 2011). The two SNPs in *FLG* present in poodles, shih tzus, and pugs were identified by direct sequencing of the gene, SNPs in *FLG* had not been tested previously in other breeds. While mutations in *FLG* have been associated with AD in some breeds of dogs, our results in the WHWT corroborate other reports of a lack of association with SNPs near *FLG* in this breed. The CanineHD SNP set did not contain a SNP in *FLG* but there was a SNP located within 20 Kb; given the long LD within dog breeds, this makes it possible but unlikely that we missed an association to *FLG*.

As canine AD is believed to be a complex disease and a GWAS for such complex diseases is looking for population-wide common variants, there is the possibility that the gene responsible for AD in a breed might not be common, but rare. The likelihood of detecting a rare variant depends on it being in linkage disequilibrium with a genotyped SNP. The Illumina Canine HD BeadChip uses equally spaced SNPs that might not capture important variation if the linkage disequilibrium degrades within two genotyped SNPs. In addition, to detect a mutation with a

small effect such as in complex traits, there is a need for higher numbers of samples to be genotyped. This number has been estimated to be between 100–300 cases and 100–300 controls for genes conferring a 3-5-fold increase in disease risk (Karlsson et al., 2007, Karlsson and Lindblad-Toh, 2008). While the population history of purebred dogs suggests diseases with a 5-fold risk are likely, association studies of human AD have been underpowered to detect a moderate effect (odds ratio of 1.5) (Karlsson and Lindblad-Toh, 2008, Barnes, 2010). The amount of time and resources necessary to collect well phenotyped samples in a single breed exhibiting a complex trait make it difficult to perform a study using that high number of affected and control dogs. In our study, while the phenotyping of the dogs was made by a careful review of each dog's history and medical records, it should be noted that control dogs were much more difficult to identify (evidenced by the lower samples collected). Indeed, AD is triggered by environmental allergens and some of the 'control' dogs might in fact be affected dogs that did not yet express the disease phenotype. We attempted to minimize this by using normal dogs over the age of five who had a greater chance of encountering the environmental trigger for the disease than a young dog. The complex nature of canine AD, involving many different and possibly interacting genetic and environmental factors is likely to account for our difficulty in mapping the AD trait.

Future studies will likely have to involve the whole-genome sequencing of a large cohort of affected and control dogs of a single breed and geographical area. The search for the elusive mutations causing AD in breeds like the WHWT shall go on!

## Acknowledgements

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