Glucoma comprises a group of heterogeneous ocular disorders that cause optic neuropathy, which results in irreversible visual impairment. Primary glaucoma (PG) is defined as a group of disorders that results in characteristic changes to the optic nerve and has no readily identifiable secondary ocular or systemic cause on routine ophthalmic examination.1–8

Canine glaucomas are classified on the basis of the possible cause (congenital, primary, or secondary), gonioscopic appearance of the drainage angle (open, narrow, or closed), stage of the

A genome-wide association study to investigate genetic loci associated with primary glaucoma in American Cocker Spaniels

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OBJECTIVE
To identify genetic associations with primary glaucoma (PG) in American Cocker Spaniels using a genome-wide association study (GWAS).

ANIMALS
A nationwide ambidirectional case–control cohort study was performed in American Cocker Spaniels that had an ophthalmic examination performed by a veterinarian. Ninety-four dogs with PG (cases) and 111 dogs without glaucoma (controls) met phenotypic criteria and had a blood sample collected after receiving informed owner consent.

PROCEDURES
Genomic DNA was extracted from whole blood samples and genotyped (CanineHD BeadChip, Illumina Inc). A case–control GWAS using a linear mixed model was performed, and 3 significance thresholds were calculated (1) using a Bonferroni correction on all single nucleotide polymorphisms (SNPs) included in the GWAS, (2) using a Bonferroni correction on only the unlinked SNPs from a pruned data set, and (3) using 10,000 random phenotype permutations.

RESULTS
Following genotype data quality control, 89 cases and 93 controls were included in the GWAS. We identified an association on canine chromosome (CFA10); however, it did not reach statistical significance. Potential candidate genes within the surrounding linkage disequilibrium interval include coiled-coil domain containing 85A (CCDC85A) and extracellular growth factor containing fibulin extracellular matrix protein 1 (EFEMP1). 

CLINICAL RELEVANCE
Primary glaucoma in the American Cocker Spaniel is a complex heterogeneous disease that may be influenced by a locus on CFA10. The candidate genes CCDC85A and EFEMP1 within the identified linkage disequilibrium interval have been shown to be involved in human open-angle glaucoma.
disease (acute, subacute, or chronic), and ultrasound biomicroscopic features of the iridocorneal angle (ciliary cleft and scleral venous plexus morphology).1,8,10

The most common type of PG in dogs is primary angle-closure glaucoma (PACG).11 An increase in PACG prevalence is noted in several dog breeds, including Cocker Spaniels, Basset Hounds, Chinese Shar-Peis, Siberian Huskies, Chow Chows, Poodles, Great Danes, Flat-Coated Retrievers, Welsh Springer Spaniels, and Bouvier des Flandres.9 Therefore, it is likely that genetics is a major contributor to the cellular and molecular mechanisms resulting in a glaucoma phenotype.1,2,12-14 In addition, sex and age play a role in the risk of developing PG, as females and middle-age to older dogs are overrepresented in most breeds.9,11,15,16

A genome-wide association study (GWAS) is an unbiased genome-wide approach that compares the genotypic profile of single nucleotide polymorphisms (SNPs) throughout the genome in cases (affected) and controls (unaffected), thus identifying genomic regions associated with a disease or trait of interest.17 In the recent past, GWASs have identified genetic intervals that are associated with PG in dogs and consequently allowed a better understanding of the underlying pathophysiology of PG. In American Basset Hounds, 3 novel genetic loci—collagen type I α2 chain (COL1A2), member rat sarcoma virus oncogene family (RAB22A), and neubulin (NEB)—have been associated with a predisposition to PACG.12,18 Another study15 using European Basset Hounds found associations with 2 different genes—ring finger protein 24 (RNF24) and pantothenate kinase 2 (PANK2) for PACG. In Dandie Dinmont Terriers, a novel locus on CFA8 was associated with PACG, with candidate genes including cutaneous T-cell lymphoma-associated antigen 5 (CTAGES), F-box protein 33 (FBXO33), leucine-rich repeat and fibronectin type III domain containing 5 (LRFNS), pinnin desmosome-associated protein (PNN), and trafficking protein particle complex subunit 6B (TRAPPC6B).2 In Border Collies, the gene olfactomedin like 3 (OLFML3) has been associated with PACG and pectinate ligament abnormality.19,20 Even though these later studies suggest that a single autosomal recessive gene in Border Collies is responsible for the development of PG, further studies are warranted to confirm this exception to the complexity of PG.19

A recent study21 of primary open-angle glaucoma (POAG) in 2 dog breeds showed that the known a disintegrin and metalloproteinase (ADAM) metallopeptidase with thrombospondin type 1 motif 17 (ADAMTS17) POAG-causing mutations were associated with height. That is, affected dogs were significantly shorter than unaffected dogs. The authors suggest that the locus underlying height may be under selection in these breeds, and also inadvertently caused an increase in prevalence of POAG.

To date, there has not been a GWAS to identify the genetic underpinnings of PG in American Cocker Spaniels, a breed that has one of the highest prevalences of PG (5.52%) when compared with the prevalence of other breed-related PG (0.89%).9 In the study reported here, we investigated the genetic basis of PG within a population of American Cocker Spaniels that was either phenotypically normal or affected by PG. Our goals were to identify regions of the canine genome associated with risk of development of PG in American Cocker Spaniels.

Methods

The study protocol was approved by the Cornell University and University of California (UC), Davis, University Institutional Animal Care and Use Committees (Cornell protocol no. 2005-0151 and UC Davis protocol no. 19044). All animal studies were conducted in accordance with the Association for Research in Vision and Ophthalmology statement for usage of animals in ophthalmic and vision research. A nationwide request was made for collaboration from all Diplomates of the American College of Veterinary Ophthalmology working in the United States and the American Spaniel Club Foundation to enroll the study population.

Animals

Blood samples were collected from privately owned American Cocker Spaniels after receiving written informed consent by the participant’s owner or legal representative.

All animals enrolled in the study had an ophthalmic examination, including diagnosis of the presence or absence of PG, performed by either a Diplomate of the American College of Veterinary Ophthalmologists (88 unaffected dogs [controls] and 82 affected dogs [cases]) or a general practitioner (23 unaffected dogs [controls] and 12 affected dogs [cases]; Supplementary Table S1).

Affected animals had no preexisting eye pathology that could result in glaucoma. All affected animals with confounding factors that could be associated with secondary glaucoma (eg, advanced cataracts, severe uveitis) were excluded from the study. Control animals were at least 8 years of age, visual, and had normotensive globes without evidence of prior elevated intraocular pressure (IOP). Ocular histopathology was available in a subset of affected animals that confirmed the clinical diagnosis of PG (Supplementary Table S1).

Sample collection and processing

Whole blood was collected in evacuated tubes containing EDTA for 94 clinically diagnosed PG cases and 111 unaffected controls. Genomic DNA was extracted from blood samples using a standard salt precipitation as follows. Blood cells were lysed using a detergent in the presence of proteinase K and incubated. Protein was precipitated from the lysate using isopropanol and purified using 70% ethanol, before being reconstituted in Tris-EDTA buffer.
Sixty-two affected and 66 control samples (n = 128 total) were processed at Cornell, and 32 affected and 45 control samples (n = 77 total) were processed at UC Davis. The DNA concentration and purity were determined by spectrophotometry (NanoDrop ND1000, Thermo Scientific), and samples were stored at ≤−20 °C until distribution for genotyping.

Genotyping

All DNA samples were genotyped with arrays (CanineHD BeadChip, Illumina Inc) containing approximately 220,000 SNPs. Twenty DNA samples from UC Davis along with the Cornell samples (n = 148) were genotyped on one version of the array (Embark Veterinary Inc), and the remaining UC Davis samples (n = 57) were genotyped on another (commercial research version, Neogen Genomics). These 2 versions of the Illumina array differed very slightly, and the CanFam3.1 assembly was used as a reference.

PC1 = Principal component 1; PC2 = Principal component 2.

Quality control on genotyping data was performed using the program PLINK (version 1.9; Chang CC, Chow CC, Tellier LCAM, Vattikuti S, Purcell SM, Lee JJ).22 PLINK files were generated from the Illumina final reports by using the TOP allele nomenclature. Samples were checked for genotype sex discrepancy with the recorded sex (using –check-sex in PLINK), and samples with missingness > 8% were removed. PLINK files from the different arrays were merged using –bmerge in PLINK before quality control steps were performed. SNPs with different base pair locations (n = 248 SNPs) in the 2 data sets were removed, as were the SNPs that had chr=0 on the Embark array but a chr number between 1 and 42 on the UC Davis array (n = 184 SNPs). SNPs with missingness > 95% and discordant SNPs were removed. This data set included 8 dogs that were genotyped twice, including 1 dog that was run on both the commercial and the Embark version of the arrays. These duplicates were used to identify 27 discordant SNPs, which were subsequently removed from the merged data. Duplicate samples and 1 sample of each pair with a high relatedness value (r > 0.60) as calculated by –genome in PLINK, were removed. This left a total of 186 dogs for analysis. SNPs in control dogs were evaluated for departure from Hardy–Weinberg equilibrium using –hardy in PLINK. This resulted in 45 SNPs with a value of P < 1 X 10^-7 that were flagged for checking after the GWAS was performed to ensure they were not highly associated.

Principal components analysis (PCA) was performed on a pruned (using –indep 50 5 2 in PLINK) set of genotypes from all dogs that passed quality control, using the program EIGENSTRAT in the EIGENSOFT package.24 PCA was done to identify outliers prior to GWAS and to look for batch effects between the 2 arrays. PCA plots were generated in R (version 4.0.2, R Foundation for Statistical Computing).25

Genome-wide association study

A case–control GWAS was performed using a linear mixed model (GEMMA, version 0.98.1; Xiang Zhou, Pjotr Prins and team).26 Only SNPs with a minor allele frequency greater than 5% and a genotyping rate greater than 90% were included. This left a total of 126,408 SNPs for analysis. A kinship matrix was included as a random effect in the model, and the Wald test was used to determine P values. A Bonferroni-corrected significance threshold (α = 0.05) was calculated using all SNPs included in the analysis, and then using only the unlinked SNPs from a pruned data set (calculated using –indep 100 10 10 in PLINK, which uses a variance inflation factor—a measure of multicollinearity in regression analyses). A permutation threshold was calculated by assigning phenotypes randomly 10,000 times, and then calculating the P value at the 5% level of the most associated SNPs.

Manhattan and QQ plots were generated in R (version 4.0.2) using qqman.25,27 The genomic inflation factor (λ), as a measure of controlling for population genetic structure, was calculated in R. Linkage disequilibrium (LD, r^2) between SNPs in associated regions was calculated using PLINK, and LD plots were generated in Jupyter notebook using Matplotlib library.24,28

Results

Principal component analysis

PCA showed no batch effect between dogs genotyped on the Embark version of the array (Cornell) versus dogs genotyped on the commercial version of the array (UC Davis; Figure 1). In the

Figure 1—Principal components analysis plots of American Cocker Spaniels with primary glaucoma (cases, red; n = 94) versus without glaucoma (controls, blue; n = 111) genotyped with versions (Embark Veterinary Inc [triangles] versus a commercial research version [Neogen Genomics, circles]) of arrays (CanineHD BeadChip, Illumina Inc) containing approximately 220,000 single nucleotide polymorphisms in a case–control study showing no batch effect between results on the basis of the version of array used. Results for 4 control dogs (blue) located at the upper end of principal component 1 (PC1) were removed as outliers before the genome-wide association study was performed. PC2 = Principal component 2.
control group, 4 outliers (individual animal results that are located separately from the main cluster) were identified on this PCA and removed, leaving 89 cases and 93 controls for GWAS. Sixty cases and 69 controls were genotyped on the Embark array; the remaining 29 cases and 24 controls were genotyped on the commercial research array. Of the 89 cases, 53 were female (60%) compared to only 43 controls (46%). This difference in the distribution of sexes between cases and controls was significant (Student 1-tailed t test, \( t = -1.88; P = .031 \)).

**Genome-wide association study**

Using 89 cases and 93 controls, we performed a case-control GWAS for glaucoma in American Cocker Spaniels and did not identify a significant association (Figure 2). The most associated SNP in this GWAS was on CFA10 at 57,267,300 bp, with a \( P \) value of \( 2.61 \times 10^{-6} \). The Bonferroni-corrected thresholds are \( 3.96 \times 10^{-7} \) and \( 1.71 \times 10^{-6} \) using all SNPs and only unlinked SNPs, respectively. The threshold calculated from 10,000 random permutations was \( 8.14 \times 10^{-7} \).

The region in LD with the most significant SNP (\( P = 2.61 \times 10^{-6} \)) at the CFA10 locus was approximately 1 megabase (MB) in length (from 56,375,894 bp to 57,267,300 bp), as defined by LD (\( r^2 > 0.2 \)) and \( P \) values within 2 orders of magnitude of the most significant SNP. This 1-MB region included the genes cilia and flagella-associated protein 36 (CFAP36), protein phosphatase 4 regulatory subunit 3B (PPP4R3B), polyribonucleotide nucleotidyltransferase 1 (PNPT1), extracellular growth factor containing fibulin extracellular matrix protein 1 (EFEMP1), and coiled-coil domain containing 85A (CCDC85A; Figure 2, Table 1).30–32

The genotypes of individual dogs at the CFA10 association showed that 90% (80/89) of affected dogs with glaucoma were homozygous for the A allele compared to only 68% (63/93) of controls (Table 2), suggesting the presence of at least 1 G allele leads to a decreased risk for PG in American Cocker Spaniels. We looked at this CFA10 SNP in 55 unphenotyped American Cocker Spaniels that were genotyped in a previous study33 and found a similar genotype frequency (AA = 74.5%, AG = 21.8%, GG = 3.6%) to the controls in our study.

Figure 2—Results of the genome-wide association study for 89 cases and 93 controls described in Figure 1. A—Manhattan and QQ plots showing the Bonferroni-corrected threshold using all single nucleotide polymorphisms (SNPs, red line) and the Bonferroni-corrected threshold using unlinked SNPs (blue line). The inflation factor (\( \lambda \)) is 1.01. B—Linkage disequilibrium plot showing the 4 megabase region around the CFA10 association. Colors indicate the amount of linkage disequilibrium (LD) with the most associated SNP, ranging from red (\( r^2 > 0.8 \)) to black (\( r^2 < 0.2 \)). The dashed vertical lines show the boundaries of the LD region of interest.
Table 1—Canine candidate genes located within the identified CFA10 linkage disequilibrium region of the dogs described in Table 2.

<table>
<thead>
<tr>
<th>Candidate gene</th>
<th>Gene name</th>
<th>Location (bp)</th>
<th>Human glaucoma reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFAP36 (ENSCAFG00000002838)</td>
<td>Cilia and flagella-associated protein 36</td>
<td>56,373,995–56,403,121</td>
<td>—</td>
</tr>
<tr>
<td>PPP4R3B (ENSCAFG00000002860)</td>
<td>Protein phosphatase 4 regulatory subunit 3B</td>
<td>56,405,121–56,467,903</td>
<td>—</td>
</tr>
<tr>
<td>PNPT1 (ENSCAFG000000046550)</td>
<td>Polyribonucleotide nucleotidyltransferase 1</td>
<td>56,478,771–56,520,992</td>
<td>—</td>
</tr>
<tr>
<td>EFEMP1 (ENSCAFG00000002919)</td>
<td>EGF-containing fibulin-like extracellular matrix protein 1</td>
<td>56,632,801–56,694,228</td>
<td>Macay et al and Collantes et al</td>
</tr>
<tr>
<td>(ENSCAFG000000048888)</td>
<td>—</td>
<td>56,888,291–56,924,212</td>
<td>—</td>
</tr>
<tr>
<td>CCDC85A (ENSCAFG00000002927)</td>
<td>Coiled-coil domain containing 85A</td>
<td>56,936,486–57,122,392</td>
<td>Athanas-Crannell et al</td>
</tr>
<tr>
<td>(ENSCAFG000000043345)</td>
<td>—</td>
<td>57,247,368–57,265,314</td>
<td>—</td>
</tr>
<tr>
<td>(ENSCAFG000000045394)</td>
<td>—</td>
<td>57,258,657–57,276,668</td>
<td>—</td>
</tr>
</tbody>
</table>

— = Not applicable. EGF = extracellular growth factor.

Table 2—Numbers and percentages of American Cocker Spaniels with (cases; n = 89) versus without (controls; n = 93) primary glaucoma, stratified by genotype class at the CFA10 association (homozygous for the A allele [AA], heterozygous for the A and G alleles [AG], homozygous for the G allele [GG], or missing) in a case–control study.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. (%) of dogs AA</th>
<th>No. (%) of dogs AG</th>
<th>No. (%) of dogs GG</th>
<th>No. (%) of dogs missing</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td>80 (89.9)</td>
<td>8 (9.0)</td>
<td>0 (0.0)</td>
<td>1 (1.1)</td>
<td>89</td>
</tr>
<tr>
<td>Controls</td>
<td>63 (67.7)</td>
<td>25 (26.9)</td>
<td>4 (4.3)</td>
<td>1 (1.1)</td>
<td>93</td>
</tr>
<tr>
<td>Total</td>
<td>143</td>
<td>33</td>
<td>4</td>
<td>2</td>
<td>182</td>
</tr>
</tbody>
</table>

Discussion

Research on glaucoma inheritance has identified several loci that are involved with specific glaucomatous phenotypes or mutations accounting for this disease in humans and dogs. However, because this is a complex genetic disease, a single mutation will not unveil the nature of PG in both species. Several SNPs have been identified and linked to PG or its endophenotypes, changing our understanding of the disease molecular pathways in humans and dogs.

In our GWAS, we identified a near-significant association on CFA10 (57,267,300 bp, P = 2.6 X 10^-6), with several potential candidate genes within the surrounding LD interval, including CCDC85A and EFEMP1. Another gene of interest that falls outside the identified LD region is vaccinia related kinase 2 (VRK2), which has been identified by linkage analysis as a candidate gene for POAG in humans. Expression of EFEMP1 is downregulated by transforming growth factor-B2, which is highly expressed in the aqueous humor of patients with POAG and may be associated with increased aqueous humor outflow resistance in POAG. Fibulins have overlapping binding sites for several basement-membrane proteins, tropoelastin, fibrillin, fibronectin, and proteoglycans. It is possible that an abnormal extracellular matrix plays a major role in aqueous humor outflow dynamics by affecting the iridocorneal angle morphology and subsequently leading toward the development of glaucoma in both humans and dogs. This hypothesis is supported by a recent study that identified different EFEMP1 coding variants that resulted in a juvenile form of POAG in humans. These gene variants caused POAG through mechanisms that involve significant intracellular protein aggregation.
and retention. In addition, the findings support the role of EFEMP1 in ocular extracellular matrix and in regulation of intraocular fluid dynamics and IOP.\textsuperscript{11} In the eye, EFEMP1 is also strongly expressed in non-pigmented ciliary body epithelium and cornea, has a minor expression in the inner nuclear layer of the retina and optic nerve head, and has a residual expression in the lens.\textsuperscript{30} Therefore, CCDC85A and EFEMP1 may enhance an individual’s risk for different forms of PG. This is in agreement with past GWASs in humans and dogs that support the complexity of this disease.\textsuperscript{17,51}

In our study, the large majority of affected dogs were homozygous for the A allele at the CFA10 association, suggesting that an increase in the number of G alleles at this SNP reduces the risk of developing PG in the American Cocker Spaniel. We did not identify any affected dogs with a genotype of GG. The high frequency of the A allele (0.886, n = 180 dogs) at the identified CFA10 SNP in this breed suggests that this region may have been selected for as part of a phenotypic selective sweep. However, from the published canine literature, we are unaware of a known signature of selection in dog breeds in the vicinity of 55 to 59 Mb on CFA10. This is the first time this locus has been associated with glaucoma in dogs.

Future research studies involving larger sample sizes are needed to validate the CFA10 locus, and follow-up studies are then needed, including variant analysis of whole-genome sequences; expression analyses; and functional and structural studies to confirm and refine the findings.

Although the limited genetic architecture and haplotype structure of domestic dogs, especially within a specific breed, allow for a GWAS with a much smaller sample size than in humans,\textsuperscript{2,12,13,52–56} a larger sample size is needed when investigating a complex disease such as PG.\textsuperscript{11} A simulation study has shown that a within-breed GWAS design, such as that used here, has more power to detect causal loci for complex diseases than an across-breed design,\textsuperscript{33} although it is more difficult to collect enough samples from a single breed to obtain the required statistical power to reach significance for a complex disease. Therefore, a larger sample size is required to validate the potential association on CFA10 in American Cocker Spaniels presented here.

Although gonioscopy was not addressed in our study, it was evaluated in nearly 50% of the affected and control dogs (data not shown). Because gonioscopic findings can be subjective, can change over time, are affected by the experience of the examiner, and the same eye can have different gradings in different regions of the iridocorneal angle, the generated data were not considered for our study. Nevertheless, although some controls were classified as having a normal open angle, all affected PG dogs that had gonioscopy performed had an abnormal angle (goniodysgenesis, narrow, or closed). This is in accordance with a PAGC study in Bouvier des Flandres, where normal dogs had a wide variation of the iridocorneal angle; however, none of the affected dogs had an open angle.\textsuperscript{57} Regardless of the iridocorneal angle morphology, given the fact that PG is usually affected by multiple genes, individuals with this disease may have unique genetic profiles that map distinct biologic routes toward the same disease.\textsuperscript{58}

According to the American Kennel club, life expectancy in American Cocker Spaniels is 10 to 14 years. To perform a GWAS in a complex disease that often occurs in middle-age to older dogs, the study design needs to consider the expected life span. Because the age (mean ± SD) for the initial presentation of PG in American Cocker Spaniels is 6.73 ± 1.13 years,\textsuperscript{9} we elected to enroll older control animals (Supplementary Table S1) to allow enrolling enough cases for a GWAS and to minimize the risk for mislabeling patients. Although it is possible that some of our control dogs may develop PG later in life, their influence on our analysis is likely to be small due to the large number of cases enrolled in this cross-sectional study.

In conclusion, this study identified CCDC85A and EFEMP1.2 genes known to be associated with PG in both humans and dogs, as possible candidates in the increased risk for development of PG in American Cocker Spaniels.

Acknowledgments

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References


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Supplementary Materials
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