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Identification by whole genome sequencing of genes associated with delayed postoperative hemorrhage in Scottish deerhounds

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Abstract

Background: Delayed postoperative hemorrhage (DEPOH) is an important health concern for Scottish deerhounds.

Hypothesis/Objectives: Identify genes associated with DEPOH in Scottish deerhounds.

Animals: Two hundred sixty-nine privately owned Scottish deerhounds.

Methods: Retrospective case-control study. DEPOH cases and controls were identified through an owner health survey. Genome-wide association analysis was performed using whole genome sequences from 8 cases and 17 controls. All cases and controls were genotyped for selected variants.

Results: Of 269 dogs, 10 met inclusion and exclusion criteria for DEPOH, while 62 controls had undergone similar surgical procedures without DEPOH. Genome-wide association analysis identified a single locus on chromosome 9 spanning 40 genes. One of these genes (*SERPINF2* encoding alpha-2 antiplasmin) was directly linked to the pathophysiology of DEPOH. The entire cohort was genotyped for a missense *SERPINF2* variant (c.605 C>T; p.A202V). Compared to dogs with the reference C/C genotype, the likelihood of DEPOH was significantly higher for dogs with the T/T genotype (odds ratio [OR] = 1235; 95% confidence interval [CI] = 23-6752; $P = 0.0005$) and with the C/T genotype (OR = 28; 95% CI = 1.4-542; $P = 0.03$).

Conclusions and Clinical Importance: *SERPINF2* is associated with DEPOH in Scottish deerhounds. Genetic testing might be able to identify dogs that are susceptible to DEPOH.

KEYWORDS
hyperfibrinolysis, postoperative bleeding, Scottish deerhound

Abbreviations: CI, confidence interval; DEPOH, delayed postoperative hemorrhage; EACA, epsilon aminocaproic acid; OR, odds ratio.

1 | INTRODUCTION

Delayed postoperative hemorrhage (DEPOH) is recognized as an important health problem in certain sighthound dog breeds, including Scottish deerhounds and racing greyhounds.[1,2] This disorder was first identified in retired racing greyhounds in 2007 based on a web-based health survey of owners across the United States. The survey results indicated that bleeding disorders were 1 of the 4 most commonly reported causes of death in this breed, with a substantial proportion of those deaths attributed to postoperative bleeding.[3]

The same research group then conducted a prospective clinical study of greyhounds undergoing routine gonadectomy.[4] This showed unexpected postoperative hemorrhage in 26% of dogs that started 36 to 48 hours after the procedure. Signs of abnormal bleeding ranged from severe skin bruising around the surgical site to frank oozing of blood from the wound. A range of assays evaluating hemostasis were performed using blood samples collected before surgery from affected and unaffected dogs. Although no defects in primary or secondary hemostasis were detected, affected dogs showed lower plasma alpha-2 antiplasmin activities compared with unaffected dogs.

The normal breakdown of fibrin blood clots (ie, fibrinolysis) is mediated by the plasma serine protease, plasmin.[5] Plasmin activity is primarily regulated through inactivation by the protease inhibitor, alpha-2 antiplasmin. Consequently, reduced alpha-2 antiplasmin activity (as was observed in affected greyhounds[4]) would predispose to enhanced fibrinolysis (hyperfibrinolysis) resulting in premature clot dissolution. Such a defect is also consistent with the delayed onset of hemorrhage after surgery (36 to 48 hours) that was observed in affected dogs.[4]

Based on a potential role for hyperfibrinolysis in the pathophysiology of this disorder, several studies have demonstrated that administration of an antifibrinolytic drug (epsilon aminocaproic acid [EACA]) is effective in preventing DEPOH in greyhounds.[6,7] The first study retrospectively examined the effect of introducing various preventive treatments, including fresh frozen plasma transfusion, EACA administration, or a combination of both therapies on the incidence of postoperative bleeding over a 5-year period in 46 greyhounds that underwent limb amputation for osteosarcoma.[6] Administration of EACA, but not fresh frozen plasma, was associated with a reduction in the incidence of bleeding. Dogs that did not receive prophylactic EACA were nearly 6 times more likely to bleed than dogs that received this drug. The second study used a prospective, placebo-controlled, randomized design to evaluate the effectiveness of EACA in preventing bleeding in 100 greyhounds undergoing routine gonadectomy.[7] Compared with placebo, EACA treatment resulted in a reduced incidence of DEPOH from 30% to 10%.

Anecdotal evidence also indicates that Scottish deerhounds are susceptible to DEPOH, with clinical signs that are similar to those described for DEPOH in retired racing greyhounds.[1] Prophylactic treatment with EACA or tranexamic acid (another antifibrinolytic drug) is recommended for both greyhounds[2] and deerhounds[1] undergoing surgical procedures. However, it is unclear whether all dogs within each of these breeds are susceptible to DEPOH and should receive prophylactic antifibrinolytic drugs. Such treatment can be expensive and could also increase the risk for drug associated adverse reactions. The long-term goal of this study is to develop a test to identify dogs that would most benefit from treatment with antifibrinolytic drugs to prevent DEPOH.

Given that DEPOH occurs in 2 breeds with high genetic relatedness (ie, greyhounds and Scottish deerhounds[8]), we hypothesized that genetic variation influences the susceptibility for DEPOH in these breeds. The objective of this study was to explore this hypothesis through case-control genome-wide association analysis (GWAA) to identify genes (and variants) associated with DEPOH in Scottish deerhounds.

2 | MATERIALS AND METHODS

2.1 | Recruitment and DNA sampling of cases and controls

Scottish deerhounds with DEPOH and matched controls were identified through a survey distributed to members of the Scottish Deerhound Club of America. A subsection of this survey requested information regarding the surgical history of the dogs, occurrence of postoperative bleeding complications, and perioperative administration of antifibrinolytic drugs (EACA or tranexamic acid). A diagnosis of DEPOH, defined as unexpected bleeding from a surgical wound starting 1 to 4 days after the procedure, was based on review of the owner's description of the event, the veterinary medical record (if available), and therapeutic response to administration of antifibrinolytic drugs (if used). Dogs were excluded if the bleeding event occurred on the same day of surgery, or after more than 4 days after surgery. Control dogs were included if they had undergone a surgical procedure where the owners reported no evidence for DEPOH. They were excluded as controls if antifibrinolytic drugs had been administered prophylactically. DNA samples from cases and controls were obtained either directly by cheek swab collection from the dogs by the owners or were archived samples obtained from the Canine Health Information Center DNA repository, or PennGen Laboratories (University of Pennsylvania).

2.2 | Whole genome sequencing

Whole genome sequencing was performed using DNA samples obtained from a subset of affected dogs (8 of 10 total) and matched controls (17 of 62 total). Information regarding these dogs is provided in Tables S1 and S2. All affected dogs with sufficient DNA quantity (>25 ng) and quality (OD 260/280 ratio = 1.8-2.0) for genomic library preparation were sequenced. Control dogs were selected at random from among those that had undergone similar surgical procedures as the affected dogs. Since a relatively small number of affected dogs were available for

sequencing, approximately twice as many control dogs were selected for sequencing in order to enhance statistical power for the association analysis.

Briefly, genomic DNA libraries were prepared using the TruSeq Nano DNA Low Throughput Library Prep Kit (Illumina, San Diego, California). Libraries were then sequenced by Novogene (Sacramento, California) on an Illumina NovaSeq 6000 to derive 150 bp paired-end short reads. For 10 of the 17 control dogs, raw sequence data (also 150 bp paired-end short reads) had been generated previously using a similar approach for 2 unrelated studies funded by the Scottish Deerhound Club. These sequences were generously shared by Dr. Kate Meurs (University of North Carolina) and by Dr. Elaine Ostrander (National Institutes of Health). Raw sequence reads were trimmed and filtered for quality using Trimmomatic (Galaxy version 0.38.1) and mapped to the CanFam4 (UU_Cfam_GSD_1.0) reference genome assembly using Bowtie2 (Galaxy version 2.4.5). After filtering of mapped reads to remove duplicates, genome coverage was estimated to average 25-fold. Mapped sequence data for all dogs were deposited in the NCBI SRA database (BioProject ID PRJNA860167). Variant calls were then made using FreeBayes (Galaxy version 1.3.5).

2.3 | Genome-wide association analysis

Genome-wide association analysis for the case-control subset of 25 dogs that were sequenced was performed using the SNP and Variation Suite version 8.9.1 (Golden Helix, Boseman, Montana). Whole genome sequence variants that met a threshold quality score of 1000 were further filtered to remove those with a call rate less than 90% (n = 1 282 522), those with a minor allele frequency less than 5% (n = 58 577 559), and those that failed Hardy-Weinberg equilibrium testing ($P < 5 \times 10^{-5}$, n = 255 196). This resulted in 4 844 960 variants for the GWAA. An efficient mixed-model association expedited statistical approach with an identity by state kinship matrix (EMMAX-IBS) was performed for additive, dominant, and recessive models of inheritance to identify associations. The statistical model was defined as $\mathbf{y} = \mathbf{X\beta} + \mathbf{Z}u + \epsilon$, where $\mathbf{y}$ explains the $n \times 1$ vector of observed phenotypes, $\mathbf{X}$ is an $n \times f$ matrix of fixed effects (f), $\boldsymbol{\beta}$ is an $f \times 1$ vector containing the fixed effect coefficients, $\mathbf{Z}$ is an $n \times t$ matrix relating the random effect (t) to the phenotype, and u is the random effect to the mixed model.[9] This model assumes residuals to be independent with an identical distribution such that $Var(u) = \sigma_g^2\mathbf{K}$ and $Var(\epsilon) = \sigma_e^2\mathbf{I}$, and such that $Var(y) = \sigma_g^2\mathbf{ZKZ}' + \sigma_e^2\mathbf{I}$.[10] For this study $\mathbf{K}$ is a matrix of pairwise genomic relationships and $\mathbf{Z}$ is the identity matrix, $\mathbf{I}$.[11] Associations of DNA variants with DEPOH were identified after a Bonferroni multiple testing correction ($P < 0.05$). Positional candidate genes were defined as genes that contained the DNA variant associated with DEPOH. To determine if the DNA variant lied within a gene, genome annotation files from CanFam4 (UU_Cfam_GSD_1.0), uuGene.txt.gz and refGene.txt.gz were accessed. These assemblies were also used to evaluate putative effects of variants on protein coding regions.

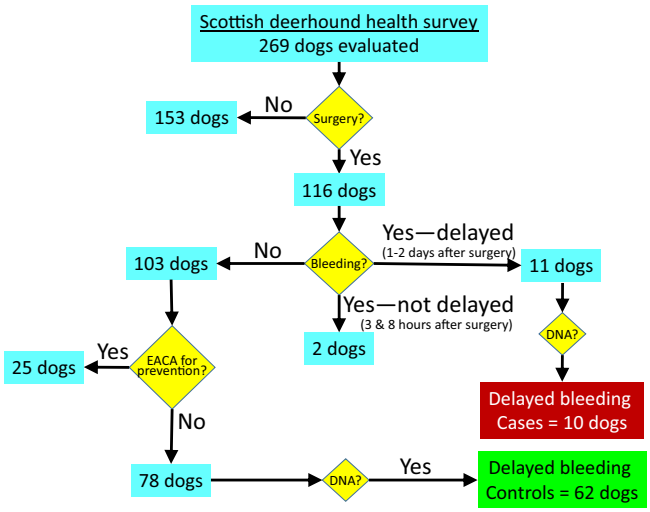

FIGURE 1 Flowchart showing the stepwise process used to identify delayed postoperative hemorrhage cases and controls for genotype–phenotype analysis. A study population of 269 Scottish deerhounds with completed health surveys was evaluated. Case inclusion criteria included a history of a surgical procedure with evidence for postoperative bleeding starting from 1 to 4 days after surgery. Cases were excluded if the bleeding occurred within 1 day or more than 4 days after surgery. Controls were included if they had a history of surgery, and excluded if they had received an antifibrinolytic drug before surgery. The final inclusion criteria for both cases and controls was the availability of DNA samples (from DNA repositories or by direct sampling of the dog) for genotyping.

2.4 | *SERPINF2* c.605 C>T and *F7* c.407 G>A genotyping

A custom allele discrimination assay (Applied Biosystems TaqMan SNP Genotyping Assay, Thermo Fisher Scientific, Waltham, Massachusetts) was developed to genotype DNA samples for the *SERPINF2* (alpha-2 antiplasmin) c.605 C>T variant. The primers and probes were 5′-ACG CTG CGG AGG TTA GAG-3′ (forward primer), 5′-CCC AGG TCC TGG CAA AGG-3′ (reverse primer), 5′-CCA GAG TCT GCA TGC AG-3′ (C-allele probe labeled with VIC dye), and 5′-CCA GAG TCT ACA TGC AG-3′ (T-allele probe labeled with FAM dye). Assays were performed according to the manufacturer's directions using a real-time PCR instrument (CFX96 Touch, Bio-Rad, Hercules, California). Assay accuracy was verified by genotyping the same case/control Scottish Deerhound DNA samples that had been sequenced using an independent whole genome approach (described previously). *F7* (coagulation factor VII) c.407 G>A variant genotype was determined by Sanger sequencing of the PCR product generated using primers 5′-ATC AAA CCT CAG CGG GGC TGG-3′ (Pri-1291) and 5′-GGG CTT GTT TCC GAG CGG G-3′ (Pri-1292).

2.5 | Statistical analysis

Case versus control allelic and genotypic odds ratios (ORs) and associated 95% confidence intervals (CIs) were calculated for the *SERPINF2*

c.605 C>T and *F7* c.407 G>A variants using data from the entire cohort according to Altman.[12] Probability (*P*) values were calculated according to Sheskin.[13] A *P* value less than 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Identification of DEPOH cases and controls

Completed health surveys from the owners of 269 Scottish deerhounds were reviewed to identify DEPOH cases and controls. A flowchart illustrating the process used to select cases and controls is shown in Figure 1. Of the original 269 dogs evaluated, 116 dogs (43%) were identified that had undergone at least 1 surgical procedure, while 16 dogs (6%) had received 2 or more surgeries. Thirteen of 116 dogs (11%) with a surgical history were reported to have experienced postoperative hemorrhage, excessive bruising, or both signs at least once after a surgical procedure. Of these, bleeding was first diagnosed less than 8 hours after surgery in 2 dogs, while bleeding or bruising was not evident until at least the morning after surgery in the remaining 11 dogs. These latter 11 dogs were considered DEPOH cases. Of those 11 case dogs, 10 dogs had DNA samples available for case-control genetic analysis.

Of the 103 dogs that had undergone surgery without evidence for postoperative bleeding, 25 dogs (24%) had received prophylactic treatment with EACA, while 78 dogs (76%) had not received any antifibrinolytic drug (including tranexamic acid). These latter 78 dogs were considered DEPOH controls. Of those 78 control dogs, 62 dogs had DNA samples available for case-control genetic analysis.

3.2 | DEPOH cases

Case information for the 10 dogs (SDH-1 to SDH-10) that met the inclusion/exclusion criteria for DEPOH are summarized in Table S1. There were 8 females and 2 males with age at the time of the surgical procedure ranging from 15 months to 10 years (median 5.5 years). Surgical procedures associated with delayed bleeding ranged from quite invasive procedures with the potential to cause extensive intraoperative bleeding, including splenectomy (SDH-2), limb amputation (SDH-4), and Caesarean section (SDH-9) to moderately invasive procedures, including ovariohysterectomy (SDH-1, SDH-7, SDH-8, and SDH-10), oophorectomy (SDH-5), and castration (SDH-3). One dog had experienced delayed bleeding after a relatively minor procedure involving removal of a large cutaneous sebaceous cyst (SDH-6).

In the majority of cases (8 of 10), signs of excessive bleeding or bruising were first recognized after the dog had returned home after surgery. Bleeding was first observed in 7 dogs on the day after surgery, while in 3 dogs bleeding was first noticed on the second day after surgery. Clinical signs indicative of unusual bleeding that were first noted by the owner or in the medical record included frank bleeding from the wound (9 dogs), excessive and often progressive bruising of the skin surrounding the wound (6 dogs), and hemoabdomen detected by abdominal ultrasound and abdominocentesis (4 dogs). Clotting function tests that were performed included measurement of prothrombin and activated partial thromboplastin times (4 dogs) and von Willebrand factor antigen (1 dog). Test results were normal in each instance. Platelet counts were available for 4 dogs. Mean (±SD) platelet counts were $193 \pm 69 \times 10^6$/L before surgery, transiently decreasing to $90 \pm 21 \times 10^6$/L between 2 to 5 days after surgery, before returning to presurgical values. All platelet counts were within the breed-specific reference range established for North American Scottish deerhounds ($37\text{-}270 \times 10^6$/L).[14] Two dogs underwent exploratory surgery to identify the possible cause of bleeding after cryptorchid castration and ovariohysterectomy surgery. However, bleeding could not be localized to a specific site in either dog.

Four dogs were treated within 12 hours of the onset of bleeding with EACA administered intravenously at doses ranging from 500 to 2000 mg. Additional EACA doses were given every 8 hours for up to 5 days in 3 dogs, while 1 dog only received a single EACA dose intravenously. Two dogs received whole blood transfusion (10 mL/kg), and 1 of those dogs also received fresh frozen plasma (10 mL/kg). Two dogs were treated with vitamin K by injection (2.5 to 5 mg/kg twice daily for 1 to 5 days) and 1 dog received Yunnan Baiyo capsules orally (500 mg every 8 hours for 5 days). Most dogs (7 of 10) also received intravenous fluid therapy. One dog experienced excessive bleeding and bruising after sebaceous cyst removal and was treated using a compressive bandage on the surgical site. Two dogs died at home before any medical treatment could be initiated. One dog was euthanized 7 days after surgery with multiorgan failure despite receiving blood transfusions and other supportive care (but no antifibrinolytic therapy). The remaining 7 dogs improved with treatment and were discharged after 2 to 5 days. Four of those 7 dogs had received an antifibrinolytic drug, while none of the 3 dogs that died had received this treatment.

3.3 | DEPOH controls

Relevant information for the 62 Scottish deerhounds identified as controls (SDH-C-1 to SDH-C-62) are given in Table S2. These included 39 female and 23 male dogs. The majority of controls had undergone surgical desexing procedures (18 castrations, 14 ovariohysterectomies, and 3 ovariectomies) or Caesarean section (16 dogs). Seven dogs were treated for major skin lacerations. The remaining 15 dogs underwent major abdominal or orthopedic procedures, including cystotomies (4 dogs), gastric dilatation/volvulus surgery (2 dogs), splenectomy (3 dogs), fracture repair (4 dogs), anterior cruciate repair (3 dogs), patellar luxation correction (1 dog), Achilles tendon repair (1 dog), and a digit amputation (1 dog). Thirteen dogs had multiple surgical procedures performed.

3.4 | Discovery of positional candidate genes by GWAA

Genome-wide association analysis was used to identify loci associated with the DEPOH phenotype in a subset of cases (8 of 10) and controls

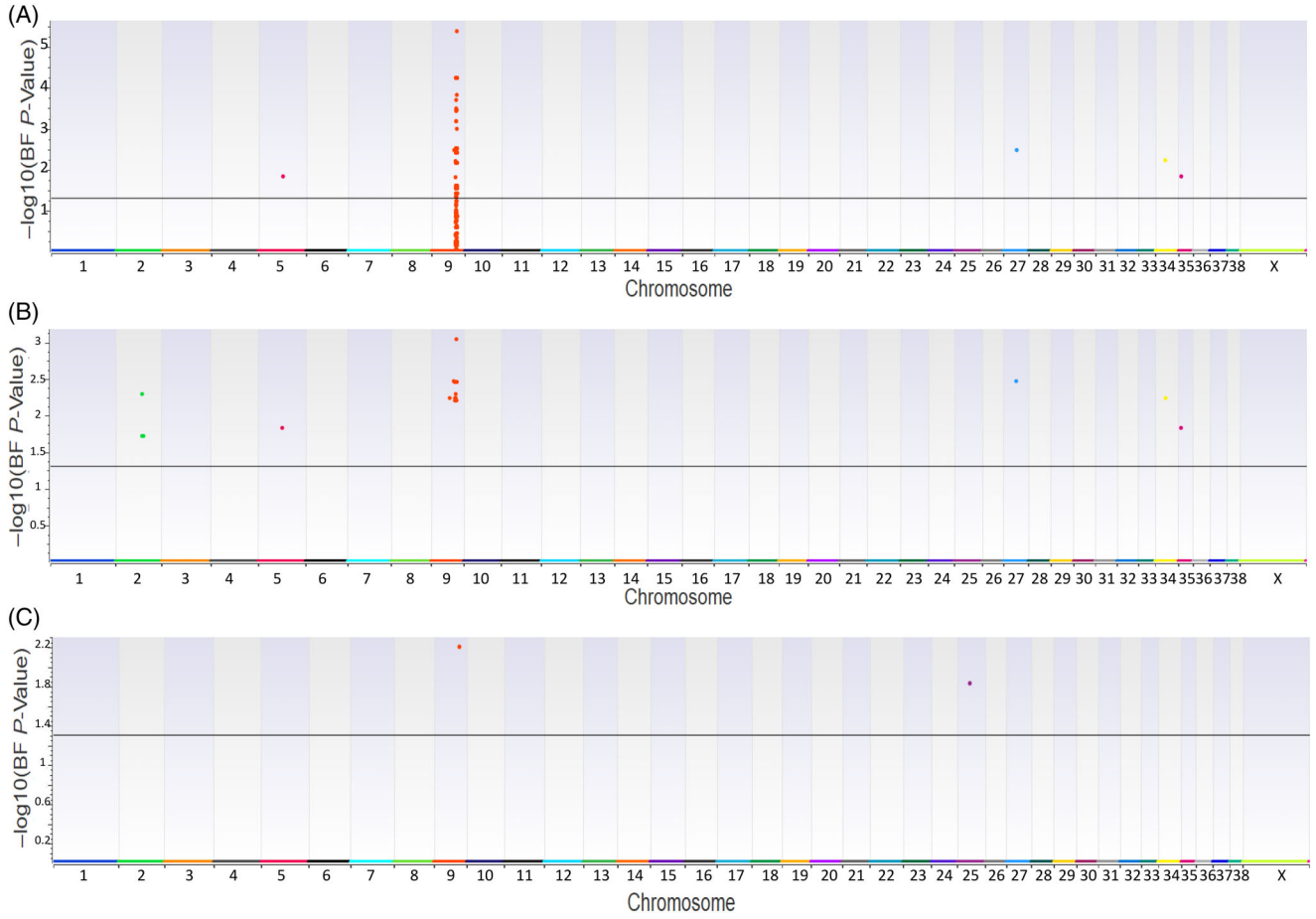

FIGURE 2 Manhattan plots identifying loci associated with delayed postoperative hemorrhage (DEPOH) in Scottish deerhounds using (A) additive, (B) dominant, and (C) recessive genetic models. Genetic variants are represented by a single dot. The relative position of each of these variants along the canine chromosomes are shown on the *x*-axis. The Bonferroni (BF) corrected −log10 transformed *P* value for the association of each variant with DEPOH is shown on the *y*-axis. Variants located above the horizontal line achieved genome-wide significance after Bonferroni correction ($P < 0.05$).

(17 of 62). As shown in Figure 2, a single locus ($D' \geq 0.95$)[15] spanning 5.5 megabases (41 to 47 Mb) was identified on chromosome 9 using an additive model that reached genome-wide significance after Bonferroni correction ($P < 5 \times 10^{-8}$). This locus contained 40 positional candidate genes. Of these, 32 positional candidate genes were protein coding and 8 were long noncoding RNA genes. Loci associated with DEPOH were also located on chromosomes 5 (44 Mb), 27 (23 Mb), 34 (17 Mb), and 35 (4 Mb) with a single positional candidate gene identified within the associated locus on chromosomes 5 (*SGIP1*), 27 (*SOX5*), and 34 (*VPS8*).

The dominant model identified 6 loci associated ($P < 0.05$) with DEPOH on chromosomes 2, 5, 9, 27, 34, and 35. With the exception of the locus on chromosome 2, all of these loci were also identified as associated with DEPOH with the additive model. However, the individual DNA variants that comprised the loci were not identical (Figure 2; Table S3). For example, on chromosome 9 the locus associated with DEPOH extended from 34 to 47 Mb (average $D' = 0.98$) and consisted of 1154 DNA variants whereas in the additive model it ranged from 41 to 47 Mb and consisted of 876 DNA variants. There were 3 variants associated with DEPOH on chromosome 2 with a $D' > 0.8$. These variants were located at 49.5 Mb ($P = 0.005$), 49.6 Mb ($P = 0.02$), and 52.1 Mb ($P = 0.02$) and were within the positional candidate genes *RNF180*, *RGS7BP* and the uncharacterized gene *LOC111092486*, respectively (Table S3).

The recessive model identified 2 loci associated with DEPOH on chromosome 9 (46 Mb, $P = 0.006$) and 25 (22 Mb, $P = 0.01$). The DNA variant that defined the recessive model locus associated with DEPOH on chromosome 9 was also associated with the phenotype in the additive model and was within an intron of the protein coding gene *SMG6* (Table S3). The DNA variant on chromosome 25 was not within a positional candidate gene.

3.5 | Positional candidate gene function

A complete listing of positional candidate genes is given in Table S3 with links to an annotation database containing information on the biological function of the orthologous human gene. After review of

TABLE 1 *SERPINF2* c.605 C>T genotype results for 72 Scottish deerhounds with known delayed postoperative hemorrhage phenotype (10 cases and 62 controls).

	SERPINF2 c.605 C>T genotype (N dogs)					
Phenotype	**C/C**	**C/T**	**T/T**	**Variant allele frequency (%)**	**Allelic OR (95% CI)**	***P*-value**
Case	0	4	6	80	10 (2.8-38)	0.0004
Control	47	15	0	12	Reference	

TABLE 2 *F7* c.407 G>A genotype results for 72 Scottish deerhounds with known postoperative bleeding phenotype (10 cases and 62 controls).

	F7 c.407 G>A genotype (N dogs)					
Phenotype	**G/G**	**G/A**	**A/A**	**Variant allele frequency (%)**	**Allelic OR (95% CI)**	***P*-value**
Case	6	4	0	20	1.4 (0.4-5.0)	0.62
Control	45	14	3	16	Reference	

this information, only 1 gene (*SERPINF2*) was identified that could be directly linked to the pathophysiology of the delayed bleeding phenotype. *SERPINF2* encodes alpha-2 antiplasmin, a serine protease inhibitor that is the primary negative regulator of plasmin mediated fibrinolysis.[16] As mentioned above, reduced plasma alpha-2 antiplasmin activity was reported in greyhounds predisposed to DEPOH.[4]

3.6 | *SERPINF2* gene variants

The DNA variants identified in *SERPINF2* were evaluated to determine whether any of the DEPOH associated *SERPINF2* variants might adversely affect gene expression based on their location within the gene and their effect on the reference mRNA (cDNA) and protein sequences. As shown in Table S4, 16 single nucleotide variants were identified, including 14 substitutions, 1 insertion, and 1 deletion. All DNA variants were in complete linkage disequilibrium. Of these, 14 were located in 5 different introns, while 2 variants (both single nucleotide substitutions) were located in exon 7. One of the exon 7 variants (c. 605 C>T) was predicted to result in an alanine to valine amino acid substitution in the protein at residue 202 (Ala202Val). The other exon 7 variant (c.696 G>A) was synonymous. Computational analysis by Polyphen-2 (PMID: 20354512) predicted that the Ala202Val substitution was likely to be benign with a score of 0.31 (sensitivity 0.90; specificity 0.89). PolyPhen-2 scores can range from 0.0 to 1.0 with higher scores considered more disruptive to protein function.

3.7 | Association of *SERPINF2* haplotype with DEPOH in the entire cohort

Since all of the identified *SERPINF2* variants were in complete linkage (ie, forming a single haplotype), the nonsynonymous *SERPINF2* c.605 C>T variant was chosen as a haplotype marker to evaluate the extent of genotype-phenotype association in the entire cohort of 72 deerhounds. As shown in Table 1, the *SERPINF2* c.605 T allele frequency in cases was over 6 times that of control dogs (allelic OR = 10; 95% CI = 2.8-38; $P = 0.0004$). None of the cases had the reference (*SERPINF2* c.605 C/C) genotype, while none of the control dogs had the homozygous variant (*SERPINF2* c.605 T/T) genotype. Compared to dogs with the reference *SERPINF2* c.605 C/C genotype, the odds for DEPOH were significantly higher for dogs with the heterozygous C/T genotype (genotypic OR = 28; 95% CI = 1.4-542; $P = 0.03$) and with the homozygous variant T/T genotype (genotypic OR = 1235; 95% CI = 23-6752; $P = 0.0005$).

3.8 | Lack of association of *F7* c.407 G>A genotype with DEPOH

Testing for *F7* c.407 G>A has been recommended by the Scottish Deerhound Club of America, in part because of the potential for association of this variant with DEPOH.[1] The *F7* gene encodes the coagulation factor VII protein. The *F7* c.407 G>A variant is a validated genetic marker for serum factor VII deficiency, which causes a mild bleeding phenotype in Beagle dogs.[17] As shown in Table 2, there was no difference in the frequency of the variant *F7* c.407 A allele compared with the reference *F7* c.407 G allele in case versus control dogs (allelic OR = 1.39; 95% CI = 0.4-5.0; $P = 0.62$).

4 | DISCUSSION

This study explores a role for genetic variation in predisposing Scottish deerhounds to DEPOH. An unbiased whole genome sequencing approach was used to identify positional candidate genes containing genetic variants that could affect the susceptibility of an individual dog to develop this condition. A strong (genome-wide) association was found for multiple variants located in a single region on chromosome 9. This region contained 40 candidate genes. As linkage disequilibrium across this locus was very strong, no single gene could be

unambiguously associated with the phenotype using this approach. Consequently, further screening of candidate genes was performed by evaluating the known function of the candidate genes within the context of the reported pathophysiology of this bleeding disorder.[4] Using this approach, *SERPINF2* (encoding alpha-2 antiplasmin) was identified as the most likely gene to be causally related to the DEPOH phenotype. This contention was supported by a prior study that showed reduced alpha-2 antiplasmin activities in greyhounds with DEPOH.[4]

Alpha-2 antiplasmin is considered the primary negative regulator of fibrinolysis through direct inhibition of the main fibrinolytic protein, plasmin.[5,16,18] The mechanism of action of antifibrinolytic drugs (EACA and tranexamic acid) currently used to prevent and treat DEPOH involves binding to the same sites on plasmin that alpha-2 antiplasmin binds.[16] Consequently, these drugs can serve as a direct artificial replacement for alpha-2 antiplasmin.

Congenital alpha-2 antiplasmin deficiency is a rare genetic disorder in humans that was originally called Miyasato disease.[18,19] Various mutations have been identified that result in varying degrees of protein loss and dysfunction. Clinical manifestations of alpha-2 antiplasmin deficiency have been systematically reviewed.[20] Homozygous deficiency typically results in serious bleeding after trauma, surgery, or dental extractions. Menorrhagia is observed in women. Intramedullary hematomas in the diaphyses of long bones, an uncommon type of bleeding, have been reported in multiple patients. Interestingly, 1 deerhound in this study (SDH-5) underwent surgery for splenic masses that were subsequently diagnosed as multiple benign hematomas. The development of these hematomas might have been exacerbated by an underlying bleeding disorder related to alpha-2 antiplasmin dysfunction.

Heterozygous alpha-2 antiplasmin deficiency in people can result in milder bleeding or no clinical signs at all.[20] In this study, 4 out of 10 affected dogs (40%), and 15 out of 62 control dogs (24%) were heterozygous for the *SERPINF2* haplotype marker. Since many but not all heterozygotes expressed the trait (ie, DEPOH), this suggests a dominant mode of inheritance with partial penetrance. The degree of penetrance could depend on a number of factors, especially those related to the potential for causing hemorrhage, such as the location or degree of invasiveness of the surgical procedure. Regardless, the likelihood of developing DEPOH in this study was much higher (by 28-fold) in dogs that were heterozygous for the *SERPINF2* haplotype marker versus dogs without this haplotype marker. The clinical implication of this finding is that *SERPINF2* heterozygous (C/T), as well as homozygous variant (T/T), dogs could benefit from administration of an antifibrinolytic drug before surgery to prevent DEPOH.

It is noteworthy that coagulation screening test results, including prothrombin time and activated partial thromboplastin times, were normal for all the Scottish deerhound cases that were tested in this study. This is consistent with findings of normal coagulation times in greyhounds with DEPOH[4] indicating that the intrinsic, extrinsic, and common coagulation pathways are unaffected by this condition. Apart from 1 dog being tested for von Willenbrand factor antigen concentrations (also normal), no other hemostasis tests were performed. Platelet counts were also normal for all dogs tested, ruling out a bleeding diathesis secondary to severe thrombocytopenia.

Alpha-2 antiplasmin deficiency would result in hyperfibrinolysis, which should be detectable using whole blood thromboelastography. However, this assay needs to be conducted on freshly collected blood, the instrumentation is not widely available, and standard protocols need to be modified to measure the rate of fibrinolysis with accuracy.[21] Alpha-2 antiplasmin deficiency should also result in loss of plasma alpha-2 antiplasmin activities. However, antiplasmin activities measured in greyhounds with DEPOH is low but not absent, and there is considerable overlap in activities between affected and unaffected dogs.[4] Consequently, other biomarkers might be needed to identify dogs affected by DEPOH.

Scottish deerhounds in the present study were also genotyped for a missense variant in the *F7* gene (c.407 G>A; p.Gly136Glu) that results in factor VII deficiency. This mutation was originally discovered in beagles[17] and this study confirms a relatively high prevalence in Scottish deerhounds. The effect of this variant on bleeding susceptibility in beagles was reported as mild to moderate.[17] Unlike *SERPINF2* c.605 C>T, the *F7* c.407 G>A variant was not associated with DEPOH in this cohort of Scottish deerhounds. This difference might be related to the differing roles of factor VII and alpha-2 antiplasmin in hemostasis. Factor VII is involved in the initiation of clot formation, while alpha-2 antiplasmin regulates plasmin-mediated dissolution of formed clots. Consequently, factor VII deficiency is more likely to result in excessive bleeding during or immediately after surgery, which would not have met our inclusion criteria for DEPOH.

Some limitations to the present study should be noted. This was a retrospective case-control study. Unlike a prospective study where standardized tests and treatments can be instituted for each case, there was reliance on the ability of owners and their veterinarians to detect and classify the clinical signs of interest appropriately. Milder indicators of DEPOH might not have been noticed or considered abnormal by veterinarians or owners resulting in affected dogs being classified as controls. Ideally all dogs should have been screened for fibrinolysis by either viscoelastic assessment of fibrinolysis or clot lysis assay. Another limitation is that we might not have identified the causal genetic variant. The *SERPINF2* haplotype marker we used in this study does alter the alpha-2 antiplasmin protein sequence. However, preliminary computational analysis indicates that this amino acid substitution is not likely to have a major effect on biological function. Consequently, this marker might only be useful for predicting the risk for DEPOH in Scottish deerhounds, and not for other breeds (such a greyhounds), which could have a different haplotype structure. Additional studies are needed to determine whether this variant, or perhaps another variant on the same haplotype, can alter alpha-2 antiplasmin expression or activities.

In conclusion, the results of this study support the hypothesis that genetic variation can influence the susceptibility to DEPOH in Scottish deerhounds. Furthermore, this variation is localized to a single region on chromosome 9 containing at least 1 gene (*SERPINF2*) that is involved in the pathophysiology of this disorder. Additional work is needed to identify a causal genetic variant within this locus. Genetic

testing has the potential to identify dogs that are susceptible to DEPOH.

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CONFLICT OF INTEREST DECLARATION

A provisional patent application (PCT/US2022/017130) based in part on this work was submitted by Washington State University with Dr Court, Dr Dillberger, and the Scottish Deerhound Club of America listed as coinventors.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All methods in this study were performed in accordance with the relevant policies of Washington State University. The use of animals, including collection of DNA samples, was reviewed and approved by the Washington State University Institutional Animal Care and Use Committee under ASAF protocols #4539 and #6714. Informed consent was obtained from all owners for participation of their dogs in this study.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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