

# Investigation of single-nucleotide polymorphisms in the *NR3C1a* glucocorticoid receptor gene in Cocker Spaniels with primary immune thrombocytopenia

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## Abstract

**Background:** In dogs, 6 single-nucleotide polymorphisms (SNPs) have been described in the glucocorticoid receptor gene *NR3C1a*, 2 of which were nonsynonymous SNPs in exons 2 and 8. The clinical importance of these SNPs is unknown.

**Objectives:** To investigate whether SNPs in *NR3C1a* are associated with clinical outcome in Cocker Spaniels with primary immune thrombocytopenia (pITP).

**Animals:** Twenty-four Cocker Spaniels with pITP presented to a referral center. Dogs were classified as slow ( $n = 11$ ) or fast responders ( $n = 12$ ) based on time required after initiating glucocorticoid treatment to achieve a platelet count  $>70\,000/\mu\text{L}$ .

**Methods:** Deoxyribonucleic acid was extracted from stored blood samples before amplification by PCR and sequencing of exons 2 and 8 of *NR3C1a*. Associations between genotype and clinical response variables were investigated.

**Results:** Neither previously identified nonsynonymous SNPs were identified. The synonymous SNP *NR3C1a:c.798C>T* in exon 2 was found at an increased prevalence compared to a previous report. No difference was found in prevalence of any genotype at *NR3C1a:c.798C>T* between fast and slow responders ( $P = .70$ ).

**Conclusions and Clinical Importance:** None of the previously reported nonsynonymous SNPs in exons 2 and 8 of the *NR3C1a* gene were detected in our cohort of Cocker Spaniels with pITP. The synonymous SNP *NR3C1a:c.798C>T* in exon 2 was reported at a higher frequency than previously, but was not associated with outcome measures that estimated responsiveness to glucocorticoids.

## KEYWORDS

canine, corticosteroids, genetics, immune-mediated, platelet

**Abbreviations:** BUN, blood urea nitrogen; DNA, deoxyribonucleic acid; EDTA, ethylenediamine tetraacetic acid; GR, glucocorticoid receptor; ITP, immune thrombocytopenia; pITP, primary immune thrombocytopenia; SNP, single-nucleotide polymorphism.

James W. Swann and Barbara Glanemann contributed equally to this study.

## 1 | INTRODUCTION

Glucocorticoids are the primary component of treatment of immune-mediated diseases in veterinary species and humans. Although their

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effectiveness is well established, adverse effects are common, and response to treatment varies among individuals.<sup>1</sup> The glucocorticoid receptor (GR) is a complex cytoplasmic receptor located in all cells.<sup>2</sup> Owing to their lipophilic nature, glucocorticoids cross cell membranes freely to bind to the GR, which then enhances or represses transcription of target genes after translocation to the nucleus.<sup>3</sup>

The GR is encoded by the *NR3C1a* gene, which has been identified in humans and dogs.<sup>2,4</sup> Polymorphisms of *NR3C1a* have been investigated in the context of various diseases in humans. Some polymorphisms in *NR3C1a* are associated with a higher risk of developing rheumatoid arthritis, whereas others correlate with decreased risk.<sup>5</sup> Polymorphisms in *NR3C1a* are associated with a more aggressive disease phenotype in multiple sclerosis and an increased likelihood of major depression.<sup>6,7</sup> Some *NR3C1a* polymorphisms in humans are associated with poor response to treatment and worse outcome in children with acute lymphoblastic leukemia.<sup>8</sup> However, GR polymorphisms were not associated with response to corticosteroid treatment in people with inflammatory bowel disease.<sup>9</sup> In dogs, 6 single-nucleotide polymorphisms (SNPs) have been described in *NR3C1a*, 2 of which were nonsynonymous SNPs, producing changes in the amino acid sequence.<sup>4</sup> These nonsynonymous SNPs were found in exons 2 and 8 of the *NR3C1a* gene.<sup>4</sup> Associations between clinical variables and presence of these SNPs have not been investigated. If associations between GR polymorphisms and clinical outcome are identified, individualized treatment strategies could be recommended based on genotype, in a similar way to selection of drugs that are substrates for P-glycoprotein in dogs according to their *MDR1* genotype.<sup>10</sup>

Immune thrombocytopenia (ITP) is diagnosed regularly in dogs presented to veterinary referral practices. Cocker Spaniels are over-represented among dogs with primary ITP.<sup>11</sup> Treatment consists of blood products and immunosuppressive treatment, with glucocorticoids used alone or in combination with other immunosuppressive drugs. In our experience, the response to glucocorticoids varies among individual dogs, with some requiring longer to regain normal platelet counts than others. There is also variability in the severity of adverse effects observed in treated dogs, with some owners perceiving glucocorticoid adverse effects to have life-limiting impact on their dogs, whereas others report no adverse effects.<sup>12,13</sup>

We aimed to investigate whether previously reported nonsynonymous and synonymous SNPs of exons 2 and 8 of *NR3C1a* are associated with clinical outcome in Cocker Spaniels with primary ITP, defined as the time taken for platelet count to return to normal after initiation of immunosuppressive glucocorticoid treatment.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample collection

Records from a university referral hospital were searched retrospectively for cases of primary ITP in Cocker Spaniels from January 2005 through December 2019. Immune thrombocytopenia was defined as

occurrence of severe thrombocytopenia (platelet count <40 000/ $\mu$ L) with no other cause identified after review of thoracic and abdominal imaging and serum biochemistry results. Travel history and testing for infectious diseases (*Ehrlichia* spp., *Anaplasma* spp., *Borrelia burgdorferi*, and *Dirofilaria immitis*; SNAP 4Dx, Idexx) were recorded. Those dogs with clinical, laboratory, or imaging findings suggestive of an underlying disease were excluded.

Data obtained from clinical records included signalment, presenting complaints, biochemical and hematologic findings, results of infectious disease testing, imaging findings, initial dosages of glucocorticoids, and concurrent treatments. The time taken for detection of a platelet count >70 000/ $\mu$ L, or a sample with platelet clumping considered to signify a similar increase, also was recorded. Typically, platelet counts were evaluated every 1 to 2 days while the dogs were hospitalized. Dogs that required  $\leq 5$  days to regain normal platelet count were classified as “fast responders”, whereas dogs that required >5 days were classified as “slow responders.” Previously described negative prognostic indicators, including presence of melena and increased blood urea nitrogen (BUN) concentration were recorded where available.<sup>11</sup>

Residual blood samples from suitable cases were obtained from an institutional blood archive, where EDTA-anticoagulated blood samples are stored routinely at  $-20^{\circ}\text{C}$  within 2 days of completion of all diagnostic tests. Informed owner consent was given for use of these residual samples, and ethical approval for use of stored samples was granted by the institutional ethics and welfare committee (URN M2018 0145).

### 2.2 | DNA extraction and gene sequencing

Red blood cell lysis and genomic DNA extraction from residual blood samples was performed by a commercial silica-based membrane system according to the manufacturer's instructions (GenElute Blood Genomic DNA Kit, Sigma, UK). Exons 2 and 8 of the *NR3C1a* gene were amplified by PCR using the primers shown in Table 1 for amplification of the nonsynonymous SNPs *NR3C1a:c.392A>G*, *NR3C1a:c.811A>T*, and *NR3C1a:c.2111T>C* and the synonymous SNP *NR3C1a:c.798C>T*.<sup>4</sup> Standard PCR amplifications were carried out by a reaction mixture of 16.8  $\mu$ L nuclease-free water, 5  $\mu$ L MyTaq buffer (Bioline, UK), 2  $\mu$ L primer mix (200 pmol/ $\mu$ L) and 0.2  $\mu$ L MyTaq polymerase (Bioline, UK) per tube, with 1  $\mu$ L of template DNA (median concentration, 92.5 ng/ $\mu$ L; interquartile range [IQR], 58.6–122.7 ng/ $\mu$ L). Reaction tubes were heated to  $95^{\circ}\text{C}$  for 3 minutes and then underwent 35 cycles of  $95^{\circ}\text{C}$  for 15 seconds,  $55^{\circ}\text{C}$  for 20 seconds, and  $72^{\circ}\text{C}$  for 30 seconds before a final incubation at  $72^{\circ}\text{C}$  for 10 minutes. The PCR products were separated by horizontal gel electrophoresis, with 2% agarose gel (Bioline, UK) containing 1.5  $\mu$ L Safe View Nucleic Acid Stain (NBS Biologicals, UK) in  $1\times$  Tris-Borate-EDTA buffer (Sigma-Aldrich, UK). The DNA was visualized under 590 nm ultraviolet light by the G-BOX Gel Imaging System (Syngene, UK). Where necessary, either gel extraction (GenElute Gel Extraction Kit, Sigma, UK) or

**TABLE 1** Primer sequences used to amplify and sequence exon 2 and exon 8 of the canine *NR3C1a* gene

Exon		Primer sequence
2	F	TGTGGCAGCTGAATAGGAG
	R*	AGGACCACTTAAACTCAGTCACA
8	F	GGCAAGACTCCAGTTAAATCC
	R*	GGAAGAAAACCAACCAAGCCAAAAC
Exon 2 sequencing primer	R	GACCCAGAAGTAAAGTCCAA

Note: F is forward primer; R is reverse primer; asterisk (\*) indicates primers used for Sanger sequencing in addition to the sequencing primer required for exon 2.

PCR clean-up (GenElute PCR Clean-Up Kit, Sigma, UK) was performed depending on the quality of the bands. The PCR products then were sent to an external laboratory (Source BioScience, UK) for Sanger sequencing. The resulting sequences were compared to the reference sequence obtained from the Ensembl gene bank (ENSCAFG00000006293), and analyzed for SNPs at the locations indicated previously,<sup>4</sup> by CLC Workbench software (QIAGEN, Denmark).

### 2.3 | Statistical analysis

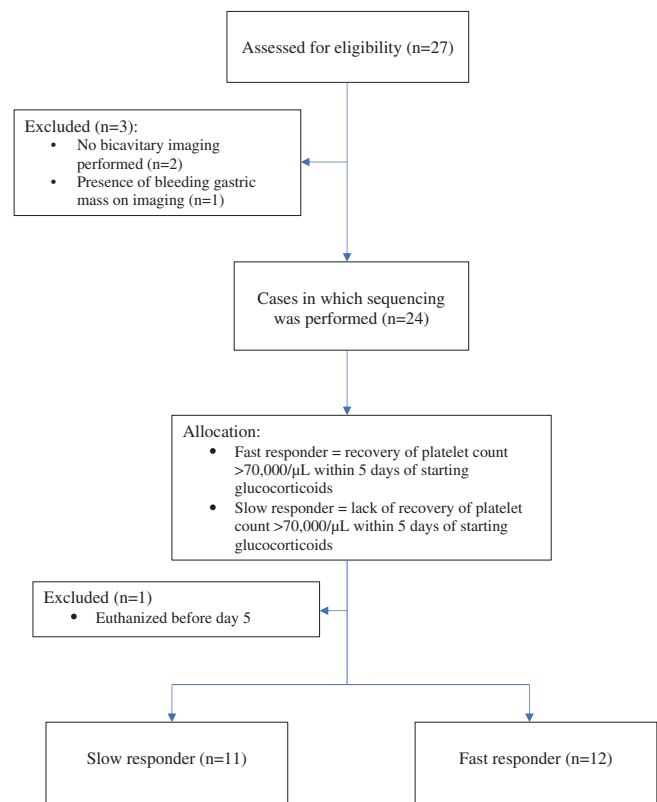
Analyses were performed by a commercial software package (SPSS, version 24, IBM). A Chi-squared test was used to compare the proportions of dogs classified as fast or slow responders according to genotype. An independent samples Kruskal-Wallis test was used to assess for differences in time to achieve a platelet count  $>70\,000/\mu\text{L}$  in dogs with different genotypes. Fisher's exact test was used to assess for differences in dogs classified as fast or slow responders, and increased BUN concentration, history of melena, vincristine treatment and monotherapy with glucocorticoids, because there were  $<5$  dogs in  $\geq 1$  groups. Statistical significance was defined as  $P < .05$ .

## 3 | RESULTS

### 3.1 | Clinical characteristics

Twenty-seven Cocker Spaniels with primary ITP were identified. Two dogs were excluded because they did not have bicavitary imaging, and 1 dog was excluded owing to the presence of a bleeding gastric mass, leaving 24 dogs for analysis. Information pertaining to signalment, history, presenting complaints and treatment can be found in Data S1, Supporting Information.

In 4 dogs, the time to regain a platelet count  $>70\,000/\mu\text{L}$  was not reached. One patient was euthanized on Day 5, and 1 on Day 11, and these patients were classified as "slow responders." One patient was euthanized a day after starting glucocorticoids, and this patient was not classified as a "fast responder" or "slow responder." One patient

**FIGURE 1** Flow diagram outlining allocation of "fast responder" and "slow responder" status based on time taken to recover platelet concentration  $>70\,000/\mu\text{L}$  in Cocker Spaniels with immune thrombocytopenia (ITP)

remained severely thrombocytopenic at Day 8 and subsequently was discharged, with incomplete follow-up information available, but this patient still was classified as a "slow responder." In the remaining 20 dogs, the median time to regain a platelet count  $>70\,000/\mu\text{L}$  was 4.5 days (IQR, 3-6 days). Based on the stated criteria, 11 dogs were classified as "fast responders" (47.8%) and 12 as "slow responders" (52.5%). The allocation of "fast responder" and "slow responder" status is summarized in Figure 1.

As shown in Table 2, no difference was found between fast and slow responders for the presence of melena ( $P = .27$ ), increased BUN ( $P = .53$ ), administration of vincristine ( $P = .14$ ), or treatment with glucocorticoid monotherapy versus multiple immunosuppressive agents ( $P = .15$ ).

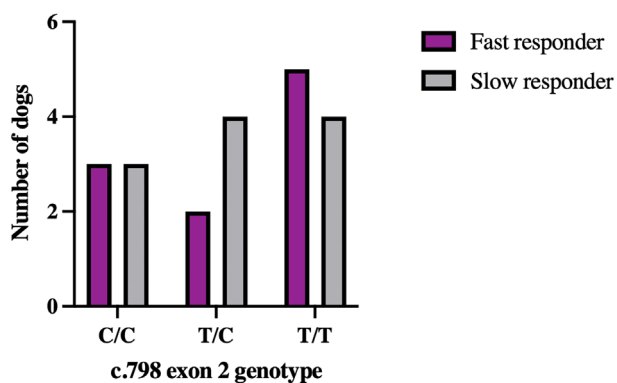
### 3.2 | Sequencing results

Sequencing results for the nonsynonymous SNP located at *NR3C1a*: c.392A>G in exon 2 were available in all dogs; all dogs were homozygous for the reference allele A. For SNPs *NR3C1a*:c.798C>T and *NR3C1a*:c.811A>T located in exon 2, sequencing failed in 2 dogs. In the remaining 22 dogs, all dogs were homozygous for the reference allele A at *NR3C1a*:c.811A>T. For the SNP at *NR3C1a*:c.798C>T, 6 dogs (27.3%) were homozygous for the reference allele C, 6 were

**TABLE 2** Distribution of various clinical variables between Cocker Spaniels with immune thrombocytopenia (ITP) classified as having a fast response to glucocorticoids (within 5 days) or slow response to glucocorticoids based on time taken to recover platelet count  $>70\,000/\mu\text{L}$

		Fast responder	Slow responder	P value
Presence of melena (N)	Yes	4	6	.27
	No	8	5	
Increased blood urea nitrogen concentration (N)	Yes	2	1	.53
	No	10	10	
Vincristine administration (N)	Yes	7	3	.14
	No	5	8	
Glucocorticoid monotherapy (N)	Yes	3	6	.15
	No	9	5	

Note: Groups compared by Fisher's exact test.



**FIGURE 2** Bar chart showing genotype frequencies for the SNP *NR3C1a:c.798C>T* (reference sequence genotype C/C) in exon 2 of the *NR3C1a* gene between Cocker Spaniels with immune thrombocytopenia (ITP) classified as having a fast response (response within  $\leq 5$  days) to glucocorticoids or slow response (response  $> 5$  days) to glucocorticoids based on time taken to recover platelet count  $>70\,000/\mu\text{L}$

heterozygous (27.3%), and 10 were homozygous for the alternate allele T (45.5%; Figure 2). Sequencing results were available for 23 dogs at the synonymous SNP location *NR3C1a:c.2111T>C* in exon 8, with all dogs being homozygous for the reference allele T.

Three dogs that were homozygous for the reference allele at *NR3C1a:c.798C>T* (C/C) were classified as slow responders and 3 as fast responders. For the heterozygous dogs (T/C), 4 were slow responders and 2 fast responders. For the dogs homozygous for the alternate allele (T/T), 4 were slow responders, and 5 fast responders. One dog homozygous for the SNP (T/T) could not be classified as a fast or slow responder because it was euthanized a day after starting to receive glucocorticoids. No significant difference in genotype frequencies was found for *NR3C1a:c.798C>T* between fast and slow responders ( $P = .69$ ), nor in time taken to regain a platelet count  $>70\,000/\mu\text{L}$  in dogs with different genotypes ( $P = .51$ ).

## 4 | DISCUSSION

We did not detect the previously reported nonsynonymous SNPs in exons 2 and 8 of the *NR3C1a* gene in a sample of English Cocker Spaniels with primary ITP. However, we did identify the synonymous SNP *NR3C1a:c.798 C>T* in exon 2, which was present at a higher frequency than reported previously but was not associated with any of the tested outcome variables that were used to estimate responsiveness to glucocorticoid treatment.

In a previous study,<sup>4</sup> which reported sequencing the *NR3C1a* gene in 97 dogs, 18 (19%) were found to be heterozygous for the nonsynonymous SNP *NR3C1a:c.811A>T* of exon 2, and 2 (2%) were heterozygous for the nonsynonymous SNP *NR3C1a:c.2111T>C* of exon 8.<sup>4</sup> Two Cocker Spaniels were present in that study population, but it was not reported whether or not those dogs had any of the SNPs. Neither of these SNPs were observed in our sample of English Cocker Spaniels, which was unexpected given the relatively high prevalence of the *NR3C1a:c.811A>T* SNP in the previous study.<sup>4</sup> Conversely, the synonymous C>T SNP at *NR3C1a:c.798C>T* in exon 2 was found at an increased prevalence in our study population, with 27.3% of dogs heterozygous and 45.5% homozygous for the alternate allele compared to 13.1% heterozygous and no homozygous dogs in the previous study.<sup>4</sup> These differences suggest that *NR3C1a* SNPs are distributed variably among breeds and geographical locations, as also observed with other genetic variation in dogs.

Synonymous SNPs cause no changes in the translated amino acid sequence, but studies in humans have shown that they may have important effects on splicing, stability, and structure of mRNA, as well as on protein folding.<sup>14</sup> Consequently, the rs6196 synonymous SNP has been associated with differences in glucocorticoid response in humans with primary nephrotic syndrome and in children with Crohn's disease.<sup>15,16</sup> However, the synonymous SNP *NR3C1a:c.798C>T* identified in our study did not appear to be associated with clinical disease outcome, suggesting that it might not be important for GR function.

Polymorphisms of the GR gene in humans have not only been associated with treatment outcomes, but also with development and severity of immune-mediated disease. The ER22/23EK polymorphism has been associated with a more aggressive disease phenotype in human patients with multiple sclerosis, and with increased predisposition to development of rheumatoid arthritis.<sup>5,17</sup> Interestingly, in the same study, some alleles also conferred decreased susceptibility to development of rheumatoid arthritis.<sup>5</sup> Although our study was aimed at investigating an association between *NR3C1a* gene SNPs and treatment outcome in ITP, it is still possible that the SNPs in the GR could be implicated in the pathogenesis of immune-mediated disease but not directly affect treatment outcome. Additional studies using control dogs not affected by immune-mediated conditions and analyses of *NR3C1a* genetic variants in other breeds could be investigated.

Because the focus of our study was the nonsynonymous SNPs identified previously,<sup>4</sup> only exons 2 and 8 of the *NR3C1a* gene were amplified, which led to identification of the increased prevalence of the synonymous *NR3C1a:c.798C>T* SNP.<sup>4</sup>

Nonsynonymous SNPs were chosen because of the resulting impact on amino acid changes, and possible impact on the function of the GR. Other synonymous SNPs were present at higher frequencies in the previous study,<sup>4</sup> for example *NR3C1a:c.2001C>A* in exon 7 of the gene in the “hunting group.”<sup>4</sup> However, because synonymous SNPs were not the intended focus of our study, additional exons were not amplified.

Our study had some limitations. First, owing to the retrospective design, enrolled dogs had received variable combinations of drugs and dosages of dexamethasone and prednisolone. Additionally, testing for anti-platelet antibodies was not available in the country where the study was performed, meaning the diagnosis of ITP was based on ruling out other causes of severe thrombocytopenia. However, we feel it is unlikely that dogs with other causes of thrombocytopenia were included in the study because previous reports indicate that severe thrombocytopenia is most commonly attributable to ITP.<sup>18</sup> Eleven dogs included in our study had no infectious disease testing performed. Although rare, *Anaplasma phagocytophilum* is present in the geographic location of the study and could not be ruled out as an underlying cause of ITP.<sup>19</sup> Owing to the retrospective nature of the study, some data were missing at some time points. For example, some dogs did not have platelet counts assessed daily, and earlier increases might have been missed. No pedigree information was available for the dogs in our study, and we could not determine whether or not dogs might have been related. This factor could be important because other shared genetic traits might have confounded our ability to detect associations between clinical variables and *NR3C1a* genotype. The Cocker spaniel breed was chosen as the sole focus of our study because of their predisposition for ITP, and to limit the impact of breed as a factor affecting frequencies of SNPs when estimating glucocorticoid responsiveness.<sup>11</sup> Finally, the small number of cases that met the inclusion criteria could mean that our study was underpowered to identify statistical differences between fast and slow responders and the presence of the identified SNP. Power calculations showed that 70 dogs would be needed to estimate the prevalence of a disorder that occurred in 20% of dogs with 0.5% acceptable margin of error at a 95% confidence interval.

In conclusion, none of the previously reported nonsynonymous SNPs in exons 2 and 8 of the *NR3C1a* gene were identified in our sample of Cocker Spaniels with pITP. The synonymous SNP *NR3C1a:c.798C>T* in exon 2 was reported at a higher frequency than in a previous report but was not significantly associated with outcome measures that estimated responsiveness to glucocorticoid treatment.

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#### CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

#### OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Informed owner consent was given for retention of residual samples, and ethical approval for use of stored samples was granted by the Royal Veterinary College ethics and welfare committee (URN M2018 0145).

#### HUMAN ETHICS APPROVAL DECLARATION

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

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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