A longitudinal study of autoantibodies against cytochrome P450 side-chain cleavage enzyme in dogs (*Canis lupus familiaris*) affected with hypoadrenocorticism (Addison's disease)

Alisdair M. Boag [a, b], Michael R. Christie [c], Kerry A. McLaughlin [d], Harriet M. Syme [e], Peter Graham [f], Brian Catchpole [a,*]

[a] *Department of Pathobiology and Population Sciences, Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire, United Kingdom*

[b] *The Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush Campus, Midlothian, United Kingdom*

[c] *School of Life Sciences, University of Lincoln, Lincoln, United Kingdom*

[d] *Oxford Centre for Diabetes, Endocrinology & Metabolism, University of Oxford, Oxford, United Kingdom*

[e] *Department of Clinical Sciences and Services, Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire, United Kingdom*

[f] *Faculty of Medicine and Health Sciences, University of Nottingham, Sutton Bonington, Leicestershire, United Kingdom*

* Corresponding author at Department of Pathobiology and Population Sciences, Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire, United Kingdom AL9 7TA

E-mail address: bcatchpole@rvc.ac.uk (B. Catchpole)

Highlights

- Twenty four percent of dogs affected with hypoadrenocorticism demonstrated autoantibodies against P450 side chain cleavage enzyme
- The autoantibody status remained consistent in most dogs, but fluctuated in some animals
- There was no relationship between autoantibody persistence and sex

Abstract

Autoantibodies directed against the P450 side chain cleavage enzyme (P450scc) have been recently described in dogs affected with hypoadrenocorticism, consistent with an immune-mediated pathogenesis of this endocrinopathy. In human autoimmune Addison's disease, autoantibodies may have a predictive value, being detectable before clinical signs developing, and have been shown to persist for a period of time after diagnosis. Furthermore, an autoantibody positive status post-diagnosis has been associated with successful remission of Addison's disease following B-cell depletion, suggesting active immunopathology in these cases. The current study was designed to investigate changes in serum P450scc autoantibody status over time in dogs diagnosed with spontaneous hypoadrenocorticism. P450scc autoantibodies were measured using a species-specific radioimmunoprecipitation assay in an initial cohort of 213 dogs, indicating a prevalence of 24%. Thirty two of these dogs had repeat samples (n = 80 in total) available for analysis. Five dogs were consistently P450scc autoantibody positive in all samples, for up to 425 days following first sampling. Three dogs were initially autoantibody positive, then became seronegative at later time points. One dog, a 1 year old female entire standard poodle, was initially negative for P450scc autoantibodies, but seroconverted 18 months after diagnosis. The remaining 23 dogs with multiple samples available were consistently P450scc autoantibody negative. Persistence was not associated with sex ($p = 0.673$). This study demonstrates persistence of P450scc autoantibodies in a

subset of dogs affected with hypoadrenocorticism and seroconversion over one year post-diagnosis. P450scc autoantibody reactivity in human autoimmune Addison's disease has been associated with sex, with females having a higher prevalence, possibly due to P450scc expression in the ovary acting as an additional source of antigenic stimulation. However, there was no sex difference in autoantibody persistence in the dogs affected with hypoadrenocorticism. Autontibody persistence in dogs with hypoadrenocorticism might represent persistent pathology, due to residual antigenic stimulation and autoimmune inflammation in the adrenal gland.

Keywords: Dog; Hypoadrenocorticism; Autoantibodies; Radioimmunoassay; Cholesterol side-chain cleavage enzyme; Addison's disease

1. Introduction

Canine hypoadrenocorticism (Addison's disease), caused by deficiency of corticosteroid hormones production by the adrenal gland, has important health and welfare implications (Scott-Moncrieff, 2015). The condition can be challenging to diagnose due to the waxing and waning clinical signs that are not pathognomonic, including lethargy, anorexia, polyuria/polydipsia, vomiting and diarrhoea (Hughes et al., 2007; Peterson et al., 1996;; Thompson et al., 2007). Hypoadrenocorticism is diagnosed by ACTH stimulation test, demonstrating a deficiency in cortisol secretory capacity (Scott-Moncrieff, 2015).

Adrenal glands histopathology from affected dogs indicates that a lymphocytic adrenalitis is present, followed by atrophy in end-stage disease (Adissu et al., 2010; Boujon et al., 1994; Frank et al., 2013; Hadlow, 1953; Schaer et al., 1986). These changes suggest an

autoimmune disorder analogous to human autoimmune Addison's disease (AAD) (Betterle et al., 2002; Mitchell and Pearce, 2012). Genetic evidence supports an autoimmune pathogenesis for canine hypoadrenocorticism, with susceptibility linked to immune response genes including MHC class II, *CTLA4* and *PTPN22* (Boag and Catchpole, 2014; Chase et al., 2006; Hughes et al., 2011; Hughes et al., 2010; Massey et al., 2013; Short et al., 2013; Short et al., 2014).

Circulating autoantibodies are regarded as an important indicator of autoimmune disease (Blizzard and Kyle, 1963; Lleo et al., 2010; Rose and Bona, 1993), autoantibodies in human AAD patients have long been recognised (Anderson et al., 1957). The primary autoantigen in human AAD is 21-hydroxylase (21-OH), with specific autoantibodies present in 90% of patients at diagnosis and in approximately 50% of patients with longer standing disease (Bednarek et al., 1992; Betterle et al., 2005; Husebye and Løvås, 2009). A case report of a hypoadrenocorticoid dog with evidence of serum 21-OH autoantibodies has been recently published (Cartwright et al., 2016), although 21-OH autoantibodies were not identified in a larger cohort of affected dogs (Boag et al., 2015). In contrast, antibodies against the cytochrome P450 side-chain cleavage enzyme (P450scc) have been described in 24% of dogs affected with hypoadrenocorticism (Boag et al., 2015), implicating an autoimmune-mediated pathogenesis in at least a proportion of cases. Autoantibodies against P450scc have also been described in human AAD, with the highest prevalence in female patients also affected with premature ovarian failure (POF), due to suspected autoimmune oophoritis (Betterle et al., 2005; Betterle et al., 1999; De Carmo Silva et al., 2000; Falorni et al., 2002; Pra et al., 2003).

The presence of adrenal autoantibodies has been shown to have a significant predictive role in a multivariate analysis of risk factors for development of human AAD (Betterle et al., 2005). In healthy humans that are positive for 21-OH autoantibodies, around 15% will subsequently develop AAD within a six year period (Husebye and Løvås, 2009). Similarly, in a cohort of 234 thyroglobulin autoantibody (TGAA) positive dogs, 20% progressed within one year to demonstrate clinicopathological evidence of thyroid dysfunction (Graham et al., 2001). Autoantibodies are also associated with continued inflammatory or autoimmune processes and their persistence may indicate active disease in the adrenal gland. Autoantibody persistence has been shown to be associated with a worse clinical outcome and progressive disease in humans with latent autoimmune diabetes of adults (Huang et al., 2016) and with an increased risk of progressing to Type 1 diabetes mellitus in children, when compared to those with reversion of autoantibodies (Vehik et al., 2016). Use of autoantibody status as a biomarker of active disease has been highlighted in a study showing clinical remission following B cell depletion, in a small open label pilot trial in humans with AAD, where one of the six patients was able to discontinue steroid therapy and had improved dynamic cortisol tests up to 27 months later (Pearce et al., 2012).

Given the potential for autoantibody testing for diagnosis and monitoring of canine hypoadrenocorticism, the aim of the present study was to document P450scc autoantibody status in a cohort of dogs affected with hypoadrenocorticism, where repeat blood sampling had been undertaken.

2. Materials and methods

2.1 Study population

The study population consisted of 213 dogs affected with hypoadrenocorticism (Boag et al. 2015). Residual serum samples were obtained following completion of diagnostic testing, undertaken either by the Royal Veterinary College (RVC) Diagnostic Service (Hatfield, UK) or NationWide Laboratories (Poulton-le-Fylde, UK). All sera included in the current study were from NationWide Laboratories.

The Royal Veterinary College Institutional Ethics and Welfare Committee approved the use of residual blood samples, taken for diagnostic purposes, for research with informed owner consent. NationWide Laboratories has approval for utilising residual clinical samples for development of diagnostic assays, provided that UK data protection legislation is observed.

2.2 Radioimmunoprecipitation assay

The autoantibody assay was carried out as previously described (Boag et al., 2015). Briefly, radiolabelled recombinant P450scc protein (20,000 CPM radioactivity; 20 µL per reaction) diluted in IMP buffer (10 mM HEPES, 150 mM NaCl, 20 mM methionine, 10 mM benzamidine, 0.01 % BSA, 2.5 mL 0.5% Triton X-100; all Sigma–Aldrich, Poole, UK) was mixed with 10 µL serum in triplicate wells and incubated in V bottomed 96-well plates at 4 °C for ~18 h. Opaque microtitre filter plates were blocked overnight with 100 µL/well of 2 mg/mL BSA in phosphate-buffered saline (PBS) and washed twice with IMP buffer prior to use. Protein A sepharose (Sigma–Aldrich, Poole, UK) was added (10 µl/well) and incubated with agitation for 20 min. The immunoprecipitate was transferred to wells of the filter plate

and washed with IMP buffer. Following drying, 100 µL/well MicroScint™ (Perkin Elmer, Cambridge, UK) was added and a Chameleon™ V plate reader (Hidex, Turku, Finland) used to quantify the amount of radioimmunoprecipitate. P450scc autoantibodies have been shown at both higher and lower dilutions and inter-assay coefficient of variation does not differ substantially with sample dilution; a representative example of dilutional parallelism is shown in supplementary figure 1.

Relative autoantibody reactivity was calculated to allow inter-assay normalisation of data as follows: $(CPM_{sample} - CPM_{negative\_standard}) / (CPM_{high\_standard} - CPM_{negative\_standard}) \times 100$. The threshold value for autoantibody positivity was set at the mean + 3 × SD of controls (n = 30) as previously described (Boag et al., 2015).

2.3 Statistical analysis

Statistical analyses were performed using SPSS© Statistics for Windows, version 20.0 (IBM Corp, Armonk, NY, USA). GraphPad Prism version 6.02 (GraphPad Software Inc., CA, USA) was used for construction of graphs. Categorical data were analysed using contingency tables, with Chi squared or Fisher's exact test used for comparisons. Continuous data was tested for normality by manual inspection of histograms, Q-Q plots and Shapiro-Wilk (Razali and Wah, 2011). For normally distributed data, comparisons were made using two-sided unpaired Student's *t*-tests, or ANOVA with post-hoc Bonferroni correction for multiple comparisons. Data not normally distributed were analysed using the Mann-Whitney *U* test or Kruskal-Wallis H test. Significance was accepted at $p < 0.05$.

3. Results

Samples from 213 dogs were previously screened for P450scc autoantibodies, with a prevalence of 24% (Boag et al., 2015) . Within this cohort, 32 dogs had between two and six repeat blood samples, with 80 samples available overall; collected between two and 787 days from the first sample being taken. The mean time from diagnosis for all samples from dogs with a known date of diagnosis, was 200 days.

Five dogs were consistently autoantibody positive in all samples analysed, up to 425 days between sampling times, these comprised one male and four female dogs, and 3 dogs (a 3 y 11 month old male entire bull terrier, a 4 y 5 month old female neutered beagle and a 2 y female entire crossbreed dog) were initially autoantibody positive, then became seronegative at later time points (Figure 1). One dog, a 1 y 8 month old female entire standard poodle, initially negative for P450scc autoantibodies, seroconverted 18 months after diagnosis (Figure 1). The remaining 23 dogs with multiple samples were consistently autoantibody negative (Table 1). Persistence of P450scc autoantibodies was not associated with sex (p = 0.673).

4. Discussion

This study represents the first longitudinal analysis of autoantibody status in dogs affected with hypoadrenocorticism and reveals that autoantibody status varies between individual dogs and at different time points post-diagnosis. Five dogs remained autoantibody positive throughout the study period. Due to the short lived nature of antibodies without continued antigenic stimulation and plasma cell production, this could be explained by the presence of long lived plasma cells in these dogs, induced during the initial autoimmune process (Amanna and Slifka, 2010). Although lymphocytic adrenalitis typically leads to adrenocortical atrophy (Boujon et al., 1994; Frank et al., 2013), it is also possible that

residual adrenalitis can persist over a prolonged period, as previously described in one dog with long standing hypoadrenocorticism (9 years) which still had evidence of mild lymphoplasmacytic adrenalitis (Frank et al., 2013). In humans, plasma cells have been associated with the source of inflammation and persistence of autoantibodies in other immune mediated diseases including SLE and rheumatoid arthritis (Radbruch et al., 2006). Autoantibody persistence can be associated with a worse clinical outcome and disease progression in autoimmune endocrinopathy (Huang et al., 2016; Vehik et al., 2016), however detailed clinical data for the dogs in this study were not available due to the retrospective nature of the sample recruitment approach.

The majority of dogs in this study (23/32) were initially, and remained, autoantibody negative. Since many of these samples were from dogs that had been diagnosed previously, an average of 200 days later, it is possible that there was a period of autoantibody positivity during the early stages of their autoimmune disease process and that these antibodies subsequently waned over time or that the immune focus in these cases is against another target in the adrenal gland such as 21-OH. Autoimmune destruction of the adrenal glands is thought to be a T cell-mediated process, and not all human patients are autoantibody positive (Husebye and Løvås, 2009). By comparison, in canine lymphocytic thyroiditis, prevalence estimates for TGAA are between 20-50%, with up to 85% reported in some breeds (Dixon and Mooney, 1999; Graham et al., 2007; Nachreiner et al., 1998; Patzl and Möstl, 2003). Whilst breed differences for P450scc autoantibodies are not particularly apparent, P450scc positivity does seem to be associated with DLA class II-type (Boag et al., 2015). Some of these dogs may not have had an autoimmune pathogenesis, neoplastic infiltration of the adrenal glands (Kook et al., 2010; Labelle and De Cock, 2005), infiltration with histoplasmosis (Frank et al., 2013) and bilateral abscessation have also been described (Korth

et al., 2008) as relatively rare causes of primary hypoadrenocorticism. It is also possible that they had secondary hypoadrenocorticism, which is not associated with lymphocytic adrenalitis (Boujon et al., 1994; Peterson et al., 1996).

Three dogs were initially P450scc autoantibody positive and reverted to autoantibody negative, which is commonly described in human AAD (Mitchell and Pearce, 2012). In dogs with lymphocytic thyroiditis, the likelihood of being TGAA positive at the time of diagnosis decreases with age, as does the autoantibody titre and those positive dogs can revert to being TGAA negative over time (Graham et al., 2007). This is likely due to diminished antigenic stimulation of B cells, following complete destruction of the endocrine tissue. This progression in the disease process, from early active inflammatory disease to atrophy of the organ (Table 2) has been suggested previously for hypothyroidism in dogs (Graham et al., 2007) and Addison's disease in humans (Mitchell and Pearce, 2012).

One dog, a female entire standard poodle, seroconverted for P450scc autoantibodies over a year after diagnosis (as evidenced in two serum samples). This might be considered unexpected in the context of adrenal pathology, as most of the cortex is typically thought to be destroyed before the onset of clinical signs, thus removing the source of antigenic stimulation. This seroconversion could represent a reactivation of memory B cells, if the dog had been previously P450scc autoantibody positive before diagnosis. Since the dog was female entire, this might represent something similar to APS type 2 in humans, or progression to premature ovarian failure, with subsequent immune-mediated oophoritis. Since the ovary expresses the greatest amount of p450scc (Luu-The et al., 2005) this tissue would be the most likely source of p450scc antigenic stimulation in an animal with adrenocortical atrophy. Autoimmune oophoritis has been described in dogs (Nickel et al., 1991), although no

additional clinical information was available for this particular dog, to allow further investigation of this line of enquiry. Further investigation of reproductive parameters in female entire dogs affected with hypoadrenocorticism is clearly warranted. Since a large proportion of dogs are routinely neutered and female entire dogs affected with hypoadrenocorticism are not usually used for breeding purposes, this could be an under-reported endocrinopathy (O'Neill et al., 2013).

The study was designed as a longitudinal cohort study with passive sample recruitment, which meant that there was no control over the cases from which samples were available, nor when those samples were taken by veterinary surgeons during routine monitoring and diagnostics. Therefore the time between first and last samples was somewhat short for some cases and, not surprisingly, no change in autoantibody status was observed. However, the lack in change over such short time periods in these cases, increases the confidence in the biological validity of the autoantibody assay and therefore strengthens the conclusions regarding persistence, reversion and seroconversion in other cases. A further limitation imposed by the sample collection strategy and availability of patient data (while conforming with UK data protection legislation), is the absence of case detail, regarding the course of the clinical disease in these dogs.

This study is the first to undertake a longitudinal assessment of P450scc autoantibody status in canine hypoadrenocorticism and demonstrates individual variability in persistence, reversion and seroconversion. Whilst P450scc autoantibodies are more prevalent in affected female dogs (Boag et al., 2015), there was no significant association between sex and persistence. Further work is required in a larger number of dogs to determine whether the presence of P450scc autoantibodies is associated with reproductive dysfunction in affected

female dogs and whether measurement of circulating P450scc autoantibodies could be of use as part of the diagnostic approach for canine hypoadrenocorticism. Furthermore, autoantibody status of a more rigorously phenotyped population would allow autoantibody status and disease severity or progression to be assessed.

Declaration of interests

The authors declare no financial or commercial conflicts of interest in relation to the content of this article. The project was part-funded by Dechra Ltd. as the industrial partner for a BBSRC industrial CASE PhD studentship, but the company played no role in the study design, data analysis, or decision to publish or preparation of the manuscript.

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Table 1. Signalment and sample information for dogs persistently P450scc autoantibody negative. Sex defined as male (M) or female (F) followed by neuter status entire (E), neutered (N) or unknown (U).

Breed	Sex	Number of samples	Days between first and last samples
Rottweiler	MN	2	2
West Highland white terrier	MN	3	22
Labradoodle	FN	4	34
Crossbreed	FN	2	787
Cocker spaniel	MU	2	42
Standard poodle	MN	2	56
Weimaraner	FU	2	13
Portuguese water dog	MU	3	111
Springer spaniel	FU	2	19
Staffordshire bull terrier	FE	2	37
Border collie	MN	2	2
Springer spaniel	FU	3	158
Bearded collie	MN	3	26
Standard poodle	FE	2	2
West Highland white terrier	FU	2	109
Labradoodle	FN	3	354
Springer spaniel	FN	3	63
Staffordshire bull terrier	FE	2	294
Crossbreed	FN	2	380
Standard poodle	MN	2	361
Border collie	FU	2	6
Crossbreed	ME	6	544
Crossbreed	MN	2	69

Table 2. General hypothetical model of autoimmune disease progression (adapted from Graham 2007 and Mitchell and Pearce 2012)

Stage	Pathology	Autoantibodies	Clinical picture
1	Focal lymphocytic infiltration	Negative (antigen release)	Normal
2	Fully developed lymphocytic infiltration	Positive	Subclinical disease
3	Majority of the target organ destroyed	Positive to negative	Overt disease
4	Atrophic gland	Negative	Overt disease

FIGURE LEGEND

Figure 1. p450scc autoantibody status over time. Time from first sample is on the x axis (days) and P450scc autoantibody reactivity, as measured by radioimmunoprecipitation.assay, on the y axis. The dotted line represents the threshold for positivity (mean + 3×SD of control sera). Dogs signalment was as follows; Dog1: FE poodle; Dog 2: ME bull terrier; Dog 3: FN beagle; Dog 4: FE crossbreed ; Dog 5: ME English springer spaniel; Dog 6: FU Dogo Argentino; Dog 7: FN crossbreed; Dog 8: FN crossbreed; Dog 9: FN Crossbreed. Sex defined as male (M) or female (F) followed by neuter status entire (E), neutered (N) or unknown (U).