Detection of circulating anti-retinal antibodies in dogs with sudden acquired retinal degeneration syndrome using indirect immunofluorescence: a case-control study.

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ABSTRACT

Sudden acquired retinal degeneration syndrome (SARDS) in dogs is proposed to have an immune-mediated etiology. However, there is conflicting evidence regarding the presence of antiretinal antibodies, as assessed by western blotting, in the serum of SARDS patients. Because of the possibility that antibodies recognize only conformational epitopes, we hypothesized that a more sensitive method to investigate circulating retinal autoantibodies in SARDS is immunofluorescence. Sera from 14 dogs with early SARDS, and 14 age- and breed-matched healthy control dogs were screened for circulating antiretinal IgG, IgM, IgE and IgA using indirect immunofluorescence on lightly fixed frozen sections of normal canine retina. Controls without canine serum were also performed. A nuclear counterstain was used to identify cellular retinal layers. Images were obtained using a fluorescent microscope, and 2-3 separate masked observers graded retinal layers for fluorescence staining intensity using a 0-3 scale. Total circulating IgG and IgM was assessed by radial immunodiffusion. Statistical analysis was performed using 2-way ANOVA, paired 2-tailed student’s t-test and correlation analysis. Intensity of IgG staining of photoreceptor outer segments was significantly higher using serum from dogs with SARDS compared with healthy controls in 2/3 observers (P < 0.05). Intensity of IgM staining throughout the retina was higher in SARDS dogs compared to matched healthy controls (P < 0.0001), although no specific retinal layer was statistically significant. There were no differences in staining intensity for IgE or IgA. Dogs with SARDS had a comparably lower circulating IgG and higher IgM than healthy controls (P = 0.01 and 0.001 respectively) and IgG and IgM were negatively correlated (r = -0.69, P = 0.007). Despite having decreased serum IgG compared with healthy controls, circulating IgG in dogs with SARDS binds photoreceptor outer segments to a greater extent. Dogs with SARDS have a relatively higher circulating IgM than matched healthy controls. The pathogenic nature of these antibodies is unknown.
1.1 INTRODUCTION

As an acute-onset blinding disease with no identifiable underlying cause, SARDS has clinical similarities to human autoimmune retinopathy (AIR). Human AIR is a group of diseases associated with circulating immunoglobulin-mediated damage to the retina resulting in acute onset visual disturbance and reduced electroretinal responses. (Weleber et al., 2005) In many cases AIR is associated with nonocular neoplasia (paraneoplastic AIR), although a subset of cases are not associated with neoplasia and are classified as nonparaneoplastic AIR. (Heckenlively and Ferreyra, 2008) Retinal thickness, particularly of the photoreceptor layer and retinal pigment epithelium is diminished in patients with AIR, (Sepah et al., 2015) but there is a notable lack of overt intraocular inflammation. (Fox et al., 2016) A definitive diagnosis of AIR is challenging, and is often presumptive based on clinical signs. (Heckenlively et al., 2010) The presence of circulating antiretinal antibodies using ELISA or western blotting, particularly to alpha-enolase or recoverin, is highly supportive of a diagnosis, and a relatively consistent finding in patients with AIR. (Grewal et al., 2014) Indirect immunofluorescence using patient-derived serum is also frequently used in the diagnosis of AIR. (Fox et al., 2016) A specific retinal binding pattern characteristic of melanoma-associated paraneoplastic AIR is serum IgG targeting inner retinal bipolar cells, (Heckenlively and Ferreyra, 2008) whereas circulating immunoglobulin (IgG) targeting photoreceptors is more characteristic of nonparaneoplastic forms of AIR. (Yang et al., 2016) Treatment of AIR commonly involves the combined use of multiple systemic immunosuppressive drugs, including steroids, antimetabolites, T-cell inhibitors, intravenous immunoglobulin and intravitreal triamcinolone acetonide. (Fox et al., 2016; Grewal et al., 2014) Treatment response is variable and is least effective in nonparaneoplastic AIR; (Ferreyra et al., 2009; Jampol and Fishman, 2009) those patients with a family history of autoimmune disease respond least favorably.

Dogs with SARDS exhibit many similar clinical signs to those of human nonparaneoplastic AIR. Although signs of endocrinopathy are present frequently in dogs with SARDS, (Oh et al., 2019) they are described infrequently in human AIR. (Breunig et al., 2013) There is no evidence that systemic neoplasia contributes to SARDS pathology. (Gilmour et al., 2006) Dogs with SARDS suffer acute onset outer retinal dysfunction (Acland and Aguirre, 1986; Acland et al., 1984; Grozdanic et al., 2007; Montgomery et al., 2008) in the absence of visible retinal pathology. There is early loss of photoreceptor outer segments (Acland et al., 1984) and apoptosis of photoreceptor nuclei. (Miller et al., 1998) Three independent studies have described thinning of the outer nuclear layer in vivo using optical coherence tomography. (Grozdanic et al., 2019; Oh et al., 2019; Osinchuk et al., 2019)
There is minimal clinical evidence of intraocular inflammation, (Miller et al., 1998) although subretinal macrophages were present early in disease in one study, (Acland et al., 1984) and a microarray-based study of acute and chronic SARDS indicated an inflammatory signature in the remaining retina. (Grozdanic et al., 2019) There is no convincing evidence of rescue of vision loss using monotherapy with immunosuppressive agents, (Komaromy et al., 2016; Stuckey et al., 2013; Young et al., 2018) although carefully controlled studies using combination therapy have not been reported.

Four studies have described the detection of antiretinal autoantibodies in patients with SARDS using either ELISA, complement fixation, western blotting, or a combination of techniques. One study found that 25% of SARDS cases had anti-neuron specific enolase antibodies compared with 0% of control animals, (Braus et al., 2008) and another study found a higher proportion of SARDS cases had antiretinal antibodies as detected by western blot, ELISA and complement fixation, compared with normal dogs, dogs with uveodermatologic syndrome and dogs with progressive retinal atrophy. (Bellhorn et al., 1988) Two studies failed to detect a difference in the presence of antiretinal antibodies between SARDS cases and unaffected dogs using either ELISA (Keller et al., 2006) or western blotting. (Gilmour et al., 2006; Keller et al., 2006) The lack of consistency in the discovery of antiretinal antibodies in SARDS compared with relative consistency in the diagnosis of human AIR is unexplained. It is possible that SARDS represents a disease that does not have an autoantibody-mediated pathogenesis. Alternatively, autoantibodies may be present to fractions of retina not previously examined such as retinal pigment epithelium, or the techniques used (ELISA or western blotting) were not capable of consistently detecting differences in autoantibodies between SARDS and healthy controls. There are three lines of supporting evidence for the latter hypothesis. Firstly, protein structure (conformational epitopes) can be altered by the techniques used for protein sample preparation in western blotting, limiting antibody binding. (Forsstrom et al., 2015) Secondly, ELISA and western blotting may not be sufficiently sensitive to detect a potentially small signal above the background from endogenous immunoglobulins present in the test tissue (most commonly retina from the same species). Thirdly, it is possible that different immunoglobulin subtypes (not examined in previous studies) such as IgA or IgE are important in the pathogenesis of SARDS. Of the four studies examining the presence of autoantibodies in SARDS, two did not specify the immunoglobulin subclass examined, (Bellhorn et al., 1988; Gilmour et al., 2006) one studied IgG alone (Braus et al., 2008) and one studied both IgG and IgM. (Keller et al., 2006)
No studies to-date have reported the use of indirect immunofluorescence in the detection of retinal autoantibodies in canine SARDS. Advantages of an immunofluorescence (immunohistochemistry) approach include optimal epitope recognition due to minimal tissue processing, and accurate subcellular visualization of specific and non-specific antigen recognition. A consensus paper on human AIR recently concluded that in order to maximize sensitivity and specificity, “the ideal assay should have a two-tier design and that western blot and immunohistochemistry should be the methods used to identify antiretinal antibodies”. (Fox et al., 2016) Considering the similarities between SARDS and AIR, there is a strong rationale to develop immunofluorescence assays to assist in the diagnosis of canine SARDS.
1.2 MATERIALS AND METHODS

1.2.1 Collection and processing of normal canine tissues

Normal canine tissues were harvested postmortem from mixed breed dogs euthanized for population control at a local animal shelter. Tissues harvested included eye, haired skin, conjunctiva and lymph node. Tissues were collected within 4 hours of death. Tissue was either immediately immersed in fixative containing 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), 3% sucrose (Sigma Aldrich, St. Louis, MO, USA) in phosphate buffered saline (PBS; Gibco, ThermoFisher Scientific, Grand Island, NY, USA) at 4 ºC, or immediately embedded for frozen sectioning unfixed (see below). Nonocular tissues were immersed in fixative for 2 hours at 4 ºC. For fixed eye processing, 3-4 slits were made in the pars plana area of the sclera, and approximately 0.5 ml of fixative was infused into the vitreous cavity prior to immersion in fixative. An eyecup dissection to remove anterior segment and vitreous was performed 2 hours after placing in fixative, and eyecups were fixed for a further 2 hours. Following fixation, tissue was briefly rinsed with PBS and immersed in 30% sucrose in PBS overnight. Tissues were subsequently briefly rinsed in PBS, minimally dried, and embedded in optimal cutting temperature medium (Tissue Tek OCT compound, Sakura Finetek, Electron Microscopy Sciences, Hatfield, PA, USA) and frozen using liquid nitrogen. Unfixed tissues were embedded in a similar manner – eyecup dissections were performed on eyes prior to embedding. Frozen blocks were stored at -80 ºC until sectioning. Sections (20 µm, CM1850 cryostat, Leica Biosystems, Buffalo Grove, IL, USA) were obtained of each tissue and two sections per slide were mounted on glass slides (TruBond 380, Electron Microscopy Sciences, Hatfield, PA) for use in optimization experiments and for use with patient serum analysis. Sections were stored at -80 ºC until staining.

1.2.2 Antibodies

Antibodies primarily conjugated to fluorophores, targeting canine immunoglobulin subclasses were selected based on validation in previous publications, and NCBI protein BLAST of manufacturer provided antigen sequence, if provided. Antibodies used included rabbit anti-dog IgG Fc Fragment specific, AlexaFluor 594 (Jackson Immunoresearch Laboratories Inc, West Grove, PA; 304-585-008), (Bizikova et al., 2011) goat anti-dog IgA FITC (Bethyl Laboratories Inc., Montgomery Tx; A40-104F), (Bizikova et al., 2014) goat anti-dog IgE
FITC (BioRad Laboratories Inc.; AHP946F) (Bizikova et al., 2014) and goat-anti-dog IgM FITC (Bethyl Laboratories Inc., Montgomery Tx; A40-116F). (Bizikova et al., 2014)

1.2.3 Immunofluorescence protocol optimization

To verify cross reactivity to canine immunoglobulins, positive control tissues were tested for each Ig subclass antibody using direct immunofluorescence. IgG and IgM were tested with normal canine submandibular lymph node, IgA was tested with normal canine conjunctiva, IgE was tested with normal canine haired skin. Because fixatives can affect the tissue binding properties of immunoglobulins by modifying conformational epitopes, (Fritschy, 2008) we compared both fixed and unfixed tissue for the intensity and specificity of staining and tissue morphology. A 1:200 dilution of antibody was used as previously published. (Bizikova et al., 2011; Bizikova et al., 2014). Slides were rehydrated with PBS at room temperature for 5 minutes, then a blocking solution (10% normal goat or rabbit serum matching the species of the antibody, Thermo Fisher Scientific, Grand Island, NY, USA in 0.1% TritonX-100, Sigma Aldrich, St. Louis, MO, USA) was applied for 30 minutes at room temperature. Antibody was incubated for 2 hours in PBS/0.1% TritonX-100 at room temperature. Slides were washed 3 times in PBS, and a final wash solution contained 1:10,000 dilution of 4',6-Diamidino-2-Phenylindole (DAPI, Sigma Aldrich, St. Louis, MO, USA). Coverslips were mounted using an antifade mounting medium (ProLong Antifade mounting medium, Invitrogen, Carlsbad, CA) and kept in the dark at 4 °C until imaging. There was no difference in staining intensity or pattern between tissue that had been previously fixed, and unfixed tissue, although cellular morphology was more optimal with fixed tissues, which was particularly notable in subsequent experiments with retina (data not shown). Representative images from fixed tissues are shown in Supplementary Figure 1 (A-D).

Subsequently, fixed haired skin was tested using an indirect immunofluorescence protocol using serum from a canine pemphigus foliaceus patient verified to have circulating antikeratinocyte IgG. (Bizikova et al., 2011) Fixed haired skin frozen sections were rehydrated, blocking solution was incubated for 30 minutes, and patient serum was incubated at 1:200 dilution in PBS/0.1% Triton X for 1 hour at room temperature. Controls omitting patient serum were run in parallel. After rinsing three times with PBS, antibody was applied (rabbit anti-dog IgG Fc Fragment specific, AlexaFluor 594 (Jackson Immunoresearch Laboratories Inc, 304-West Grove, PA; 304-585-008)) (Bizikova et al., 2011) at 1:200 dilution in PBS/0.1% Triton-X for 2 hours. Slides were rinsed,
counterstained with DAPI and mounted as previously described. Fluorescence microscopy images were taken (MFR) at 40x magnification. Representative images of slides incubated without patient serum (Suppl. Fig. 1E) and with patient serum (Suppl. Fig 1F) are shown, demonstrating positive staining in the basal epidermal layer in the patient with pemphigus foliaceus.

1.2.4 SARDS patient population and serum isolation

Patients with SARDS were prospectively recruited, and subsequently, control dogs with normal ophthalmic parameters, matched by breed and age, were recruited (matched healthy controls). The dogs recruited comprised part of the population described in a recent publication with identical inclusion and exclusion criteria. (Oh et al., 2019) Inclusion criteria for the SARDS patients were vision loss of less than 6 weeks’ duration, a clearly visible fundus using ophthalmoscopy, and a confirmed diagnosis of SARDS (history, minimal fundus evidence of retinal degeneration with severe attenuation of ERG a- and b-wave amplitudes, consistent chromatic pupillary light reflex findings). Inclusion criteria for the matched control dogs were consistent age- and breed to a matched SARDS patient, a clearly visible fundus using ophthalmoscopy, normal ERG a- and b-wave waveforms and amplitudes. Exclusion criteria for both the SARDS and control groups included systemic administration of immunosuppressive medications within the previous 21 days, the use of a home-cooked (non-commercial) diet, and any prior history of systemic or ocular neoplasia, uveitis, glaucoma, mature or hypermature cataracts, intraocular surgery, inherited retinal disorders, liver or kidney failure, and pre-existing endocrinopathy. All animal procedures were conducted in adherence to the guidelines outlined in the Animal Welfare Act, and with Institutional Animal Care and Use Committee review and approval (IACUC approval number 15-132-O), NC State University Veterinary Hospital board approval and client written informed consent. All patients visited the NC State University Veterinary Hospital for evaluation. Patients and controls were fasted for the examination, and full ophthalmic examination, optical coherence tomography, electroretinography, physical examination, blood testing (serum biochemistry, complete blood count) and urine testing (urinalysis and urine creatinine levels) were performed. Results of these tests are provided in a previous manuscript. (Oh et al., 2019) Whole blood (4ml in a plastic clotting tube; BD vacutainer, Fisher Scientific, Waltham, MA, USA) was collected for serum antibody evaluation, and allowed to clot at room
temperature for 30 minutes. Blood was centrifuged in a spin-out rotor at 1500 g for 10 minutes. Serum was removed from the compacted red blood cells, aliquoted and frozen at -80 °C until assay.

1.2.5 Indirect immunofluorescence using SARDS and matched healthy control serum

Serum from all 28 dogs was used with the optimized protocol. Slides were stained in batches of 12; all 4 Ig subclasses (IgG, IgM, IgA and IgE) were assayed in one batch, using serum from one dog with SARDS, its matched healthy control, and a parallel control slide with no patient serum and each secondary antibody only. Slides were stained using a commercial staining rack (Shandon Sequenza Immunostaining Center, Fisher Scientific, Grand Island, NY, USA). The optimized protocol used for all 28 patients was as follows. Slides containing normal canine retinal sections were dried for 5 minutes at room temperature, and rehydrated with PBS. Blocking solutions were used that represented the same species as the secondary antibody: normal goat serum (IgM, IgA, IgE) and normal rabbit serum (IgG), and serum was diluted to a 10% solution in 0.1% triton X-100 in PBS. Blocking solution was applied for 30 minutes at room temperature. Patient serum was applied to sections for 1 hour at room temperature, diluted to 1:200 in PBS/0.1% Triton X-100. No patient serum controls were incubated with 0.1% Triton X-100 in PBS. After 3 washes of 5 minutes each with PBS, secondary antibody was applied for 2 hours at 1:200 dilution in PBS/0.1% Triton X-100. Slides were rinsed, counterstained and mounted as previously described.

1.2.6 Imaging and quantification

Images were taken at 40x magnification (IX73 inverted fluorescence microscope with cellSens software, Olympus Life Science, Waltham, MA, USA); 4 images were taken of each slide. The exposure duration of each antibody subclass was determined using the no patient serum control slide, determining an exposure that resulted in detectable but minimal visible fluorescence from all retinal layers. Slides incubated with SARDS and healthy control serum were imaged using the same exposure duration. A Z-stack encompassing all retinal layers was taken at 0.88 µm step size. A Z-projection was generated from this stack, and used in image analysis.

Image analysis was performed by two independent masked observers. Observers were trained on the orientation of retinal layers and grading system prior to analysis. Because of disparity in the result between the two graders for IgG analysis, a third masked observer graded the IgG images only. Sections were graded for
fluorescence intensity as previously described, (Bizikova et al., 2011) with a modified grading system for each retinal layer: RPE (retinal pigment epithelium), OS (photoreceptor outer segment), IS (photoreceptor inner segment), ONL (outer nuclear layer), OPL (outer plexiform layer), INL (inner nuclear layer), IPL (inner plexiform layer), GCL (ganglion cell layer), NFL (nerve fiber layer) and vitreous. Fluorescence intensity was graded on a 0-3 scale (0 = absent, 1 = mild, 2 = moderate, 3 = severe) for each layer. Statistical analysis was performed comparing the intensity of fluorescence using serum from dogs with SARDS and matched healthy controls, removing signal intensity from the no serum control for each immunoglobulin subtype (SARDS minus no serum and healthy control minus no serum respectively).

1.2.7 Radial Immunodiffusion assay for total immunoglobulin quantification

Radial immunodiffusion (RID) assay kits were purchased from a commercial supplier (Triple J Farms, Bellingham, WA, USA) for canine IgG (product number: A28411) and canine IgM (product number: A03411). The RID assay has been previously validated in our clinical immunology laboratory and follows the manufacturer’s instructions (Fogle, personal communication). Briefly, assays were performed with room temperature reagents. Patient serum samples were thawed and kept on ice until use. Samples and standards were run in duplicate, and a 5 µl sample volume was used for IgG, a 3 µl sample volume for IgM. IgM samples were incubated at room temperature for 48 hours, IgG samples for 24 hours prior to measuring as end-point readings. The precipitin ring diameter was determined by viewing the well over an indirect light box with a fine scale magnifying comparator using a reticule with 0.1mm divisions. The diameter was measured in duplicate for each ring. The patient samples were compared to a standard curve. The standard curve was generated using samples of known concentration and following the manufacturer’s instructions for endpoint readings. The average zone diameter for a well was squared and plotted against the standard concentrations with a linear regression to determine the standard curve. Samples that exceeded the maximum value for the reference range standards were diluted 1:1 in PBS and re-assayed.

1.2.8 Data analysis
All statistical analysis was performed in a computerized statistical analysis program (GraphPad Prism version 5.0a for Mac, GraphPad Inc, San Diego, CA, USA). Data is presented as mean ± standard error of the mean (SEM).

For immunofluorescence grading results, a two-way ANOVA was used to compare mean grade (0-3) for each immunoglobulin subclass. The two independent factors in the two-way ANOVA were disease status (SARDS or healthy control) and retinal location (individual layers from RPE to vitreous). A Bonferroni post-test was used to identify layer-specific differences between dogs with SARDS and matched healthy controls.

For patient demographics, clinical pathology findings and radial immunodiffusion assay IgG or IgM results (before and after correction for protein concentration), a two-tailed paired student t-test was performed comparing dogs with SARDS with their matched healthy controls. The relationship between circulating IgG and IgM was correlated using a Spearman correlation, as data were not determined to be normally distributed using a D’Agostino and Pearson omnibus normality test ($P < 0.0001$). The correlation between corrected circulating IgG and IgG immunofluorescence staining intensity of the photoreceptor outer segments was correlated with a Pearson correlation, as data were normally distributed for all masked graders ($P = 0.17-0.70$). The correlation between corrected circulating IgM and total retinal IgM immunofluorescence staining intensity was correlated with a Pearson correlation, as data were normally distributed for both masked graders ($P = 0.67$ and 0.76). Linear regression was used to determine correlation and best fit line between a demographic variable (age, time since vision loss) and serum protein-corrected circulating IgG or IgM.

The sensitivity and specificity of serum protein-corrected circulating IgG and IgM was estimated by calculation of the area under the curve of receiver operating characteristic as previously described. (Oh et al., 2019) The AUC is classified as follows: 0.9 to 1.0 = excellent, 0.80 to 0.89 = good, 0.70 to 0.79 = fair, 0.60 to 0.69 = poor, and 0.50 to 0.59 = worthless. (Metz, 1978)
1.3 RESULTS

1.3.1 Participants

Dogs with SARDS (n = 14) within 6 weeks of vision loss, and age- and breed-matched healthy control dogs (n = 14) were recruited. Table 1 describes the demographics of the dogs with SARDS and matched healthy controls. Dogs with SARDS had undetectable a- and b-waves on ERG, compared with normal a- and b-waves in matched healthy controls, as previously described in a publication on the same group of animals. (Oh et al., 2019) Dogs with SARDS had significantly higher body condition score, serum total protein, albumin and globulin, plasma protein, white blood cell count and segmented neutrophil count than the matched healthy control dogs (paired student’s t-test; Table 1).

1.3.2 Detection of circulating anti-retinal antibodies

Statistical analysis of immunofluorescence is shown in Table 2. In dogs with SARDS, there was consistent IgG labeling of a retinal layer corresponding to the proximal retinal outer segment region of the photoreceptors (Fig. 1A), which was not detected in matched healthy controls (Fig. 1B). All three graders found the majority of case-pairs had a higher overall fluorescence intensity in dog with SARDS compared with matched healthy controls (Table 2). Using 2-way ANOVA, masked grader 1 (Fig. 1C) identified a significant variation with the variable retinal location, but not with either or the interaction (Table 2). Bonferroni post-test revealed that the only retinal layer that was significantly different between dogs with SARDS and healthy controls was the photoreceptor outer segment area (P < 0.01, Table 2). Masked grader 2 (Fig. 1D) also identified a significant variation with the variable retinal location, and also with disease, but not with the interaction (Table 2). Bonferroni post-test for this grader did not identify any retinal layer that was significantly different between SARDS and healthy control sera (P > 0.05, Table 2). A third masked (board-certified ophthalmologist) grader also evaluated the images (Fig. 1E) and had similar findings to grader 1: with 2-way ANOVA, grader 3 found a significant variation with the variable location, but not with the interaction (Table 2). With the Bonferroni post-test, the only retinal layer that had significantly higher fluorescence intensity in dogs with SARDS was the photoreceptor outer segments (P < 0.05, Table 2).
Using IgM immunofluorescence staining of serum from dogs with SARDS, there was an overall higher level of retinal fluorescence (Fig. 2A), compared with matched healthy controls (Fig. 2B). Both graders found the majority of case-pairs had a higher overall fluorescence intensity in dog with SARDS compared with matched healthy controls (Table 2). For both masked graders 1 (Fig. 2C) and 2 (Fig. 2D), two-way ANOVA showed a significant effect of location and disease but not the interaction (Table 2). With Bonferroni post-test, no individual layer had any difference between SARDS and controls ($P > 0.05$ for all).

Using IgA retinal immunofluorescence staining, there was overall no notable difference in fluorescence between sera from dogs with SARDS (Fig. 3A) and matched healthy controls (Fig. 3B). For both masked graders 1 (Fig. 3C), and 2 (Fig. 3D), two-way ANOVA did not identify any significant differences between groups for location, disease or the interaction (Table 2).

Using IgE retinal immunofluorescence staining, there was overall no notable difference in fluorescence between sera from dogs with SARDS (Fig. 3E) and matched healthy controls (Fig. 3F). For both masked graders 1 (Fig. 3G) and 2 (Fig. 3H), two-way ANOVA did not identify any significant differences between groups for location, disease, or the interaction (Table 2).

### 1.3.3 Quantification of total circulating IgG and IgM

Radial immunodiffusion assay was performed on sera from dogs with SARDS and matched healthy controls. Total circulating IgG did not differ significantly between dogs with SARDS ($2378 \pm 183$ mg/dL; 0/14 samples exceeded the published adult dog upper limit of 5000 mg/dL (Poffenbarger et al., 1991)) and matched healthy controls ($2728 \pm 124$ mg/dL; 0/14 samples exceeded the published adult dog upper limit; 11/14 dogs with SARDS had a lower total IgG compared with matched controls; $P = 0.06$, Fig. 4A) but total IgM was significantly higher in dogs with SARDS ($419 \pm 51$ mg/dL, 2/14 samples exceeded the published adult dog upper limit of 580 mg/dL (Poffenbarger et al., 1991)) compared with matched healthy controls ($255 \pm 16$ mg/dL; 0/14 samples exceeded 580 mg/dL; 12/14 dogs with SARDS had a higher total IgM compared with matched controls $P = 0.006$, Fig. 4B). Serum total protein was significantly higher in dogs with SARDS ($6.7 \pm 0.16$ g/dL) compared with matched healthy controls ($5.9 \pm 0.08$ g/dL; $P = 0.009$, Fig. 4C), as were serum albumin and plasma total protein, but not hematocrit (Table 1). A previous study found that water deprivation in dogs causes increases in plasma protein concentrations, commonly without alteration in hematocrit (Hardy and Osborne,
1979), therefore we hypothesized the increases in serum protein concentration reflected mild dehydration and a more concentrated serum. We subsequently recalculated serum immunoglobulin as a ratio (immunoglobulin content divided by serum total protein, both values in mg). When serum total protein of each individual dog was accounted for, serum IgG was significantly lower in dogs with SARDS (0.35 ± 0.03) compared with matched healthy controls (0.46 ± 0.02; \( P = 0.001 \), 12/14 dogs with SARDS had a lower IgG than matched healthy controls; **Fig. 4D**). Area under the receiver operating curve was 0.79 (fair, \( P = 0.009 \) ratio < 0.376 has sensitivity of 57% and specificity of 93%). Serum IgM remained significantly higher in dogs with SARDS (0.062 ± 0.007) compared with matched healthy controls (0.043 ± 0.003; \( P = 0.01 \), 10/14 dogs with SARDS had a higher IgM than matched healthy controls; **Fig. 4E**). Area under the receiver operating curve was 0.78 (fair, \( P = 0.012 \), ratio > 0.052 has sensitivity of 50% and specificity of 93%). Overall, there was a significant negative correlation between IgG and IgM (Spearman \( r = -0.45 \), \( P = 0.017 \), **Fig. 4F**). When separated by disease, however, the correlation was only significant for dogs with SARDS (Spearman \( r = -0.69 \), \( P = 0.007 \)) but not for healthy controls (Spearman \( r = 0.05 \), \( P = 0.85 \)).

Small n-numbers precluded statistical analysis of circulating immunoglobulins by breed, although no large variations were noted between or within breeds for either IgG or IgM (data not shown). For dogs with SARDS, there was no significant correlation between time (days) since the onset of vision loss and either IgG (\( R^2 = 0.002 \), \( P = 0.87 \)) or IgM (\( R^2 = 0.08 \), \( P = 0.33 \)). There was also no significant correlation in dogs with SARDS between age (years) and either IgG (\( R^2 = 0.03 \), \( P = 0.86 \)) or IgM (\( R^2 = 0.009 \), \( P = 0.74 \)). There was no significant correlation between circulating IgG and photoreceptor outer segment fluorescence intensity in sera from dogs with SARDS from any of the three graders (grader 1 Pearson \( r = -0.21 \), \( P = 0.46 \), grader 2 Pearson \( r = -0.18 \), \( P = 0.54 \), grader 3 Pearson \( r = -0.09 \), \( P = 0.77 \)). There was no significant correlation between circulating IgM and overall retinal fluorescence intensity in sera from dogs with SARDS from either of the two graders (grader 1 Pearson \( r = -0.07 \), \( P = 0.80 \), grader 2 Pearson \( r = -0.4 \), \( P = 0.16 \)).
1.4 DISCUSSION

We have identified specific immunoglobulin signatures unique to canine SARDS. Dogs with SARDS have decreased serum IgG compared with age- and breed-matched healthy controls, yet circulating IgG binds more readily to antigens in the photoreceptor outer segment region. Dogs with SARDS also have an increased circulating IgM, and IgM overall has a greater affinity to the retina than healthy control dog serum IgM. Circulating IgG and IgM were negatively correlated in dogs with SARDS. These findings indicate potential systemic immune dysregulation in canine SARDS that warrants further study.

These findings confirm the presence of circulating antiretinal antibodies in the majority of dogs with SARDS. Although both IgG and IgM bound more readily to retinal tissue in dogs with SARDS, photoreceptor-specific binding was only noted with IgG. In contrast, IgM had a more generalized binding pattern. The IgG binding site correlates with a previous study describing loss of photoreceptor outer segments in acute SARDS. (Acland et al., 1984) Although we acknowledge that our sample size of 14 dogs in each group is smaller than many studies in human AIR, (Ten Berge et al., 2018) our work can begin to draw certain parallels between canine SARDS and human AIR. Similar to our findings in SARDS, human AIR is also characterized by the presence of circulating immunoglobulins targeting retinal antigens. The pathophysiology of human paraneoplastic AIR is relatively clear: antibodies to shared antigens between the neoplasm and retinal cells contribute to the retinal pathology. (Duvoisin et al., 2017; Yang et al., 2016) Circulating antibodies can cross the plasma membrane and access intracellular epitopes, (Xiong et al., 2013) and photoreceptors have been shown to uptake antibodies by endocytosis, leading to caspase-mediated apoptosis. (Shiraga and Adamus, 2002) The pathophysiology of nonparaneoplastic AIR is less clear, with many proteins targeted by antiretinal antibodies not defined. (Sen et al., 2017) One major subject of debate in human nonparaneoplastic AIR is whether the autoantibodies are causative or consequential to the retinal degeneration. Antiretinal antibodies have been identified in normal humans, (Shimazaki et al., 2008) patients with systemic autoimmune disorders, (Lee et al., 2009) and in patients with forms of inherited retinal degeneration. (Heckenlively et al., 1999) Additionally, in one study less than half of human patients with signs and clinical findings consistent with AIR had detectable antiretinal antibodies. (Adamus et al., 2004) Many dogs with early SARDS have small retinal detachments visible on optical coherence tomography imaging (Grozdanic et al., 2019; Oh et al., 2019; Osinchuk et al., 2019) indicating a potential breakdown of the blood-retinal barrier, and subretinal macrophage
invasion is also present histologically. (Acland et al., 1984) It is possible that breakdown of the blood-retinal barrier occurs as a consequence of acute SARDS, resulting in circulatory access and subsequent retinal autoantibody production to previously unrecognized retinal antigens. Further studies are necessary to determine the pathogenic nature of antiretinal autoantibodies in dogs with SARDS before a definitive conclusion is made.

The reason for the relatively higher frequency of detection of antiretinal antibodies in our study compared with previous studies (Gilmour et al., 2006; Keller et al., 2006) warrants discussion. It could be partly explained by the different methodologies used for detection of antibodies. Western blotting has limitations on the detection of very large or very small proteins, due to difficulty in transfer of these proteins from the gel to the membrane. (Smejkal and Gallagher, 1994) Western blotting can also only reliably detect proteins with moderate to high water solubility, and sample preparation methodology significantly affects protein detection. (Bass et al., 2017) The retinal photoreceptors are particularly lipid-rich, (Fliesler and Anderson, 1983) therefore standard western blotting techniques may not represent the most optimal method of detection of autoantibodies targeting photoreceptors. It is also possible that autoantibody production is targeted not against protein, but phospholipids, as has been described in human antiphospholipid syndrome (APS), in which patients present clinically with autoimmune disorders, endocrinopathies, and in some cases visual deficits. (Bourgault et al., 2015; Breunig et al., 2013) ELISA using specialized plastic coated with specific phospholipids is utilized in the diagnosis of APS, (Roggenbuck et al., 2012) and these specialized ELISA techniques were not used for SARDS autoantibody detection, (Keller et al., 2006), which might explain the lack of detection. In addition, it is difficult to differentiate endogenous antibodies present within the test tissue from patient derived antibody using either ELISA or western blot. Immunofluorescence using frozen tissue sections offers advantages over ELISA or western blotting in two main ways: firstly, it allows detection of autoantibodies to any cellular component including proteins and lipids, and secondly, the tissue localization of antibody binding can help to differentiate background labeling from specific labeling. Therefore, immunofluorescence may represent a more sensitive method to detect autoantibodies in canine SARDS. However, similar to the recent recommendations in the diagnosis of human AIR, (Fox et al., 2016) it will likely be advisable to combine at least two diagnostic methods such as immunofluorescence and western blot, and also to validate these
methods using appropriate controls and established methodology. (Forooghian, 2016) Our findings initiate the process to determine the importance of antiretinal antibodies in SARDS pathology.

Our work also indicates potential systemic immune dysregulation in dogs with SARDS. We identified a comparably higher IgM and lower IgG in the majority of dogs with SARDS compared with matched healthy controls, although IgG and IgM concentrations were predominantly within published normal adult dog ranges for both controls and dogs with SARDS. (Poffenbarger et al., 1991) The significant negative correlation in dogs with SARDS and not in controls indicates this might be a useful diagnostic criterion and suggests systemic immune dysregulation in these dogs. Similar findings are present in human hyperimmunoglobulin M syndrome (HlgM): patients have defects in the immunoglobulin class switch recombination from IgM and are therefore characterized by low or absent circulating IgG, IgA or IgE, and either elevated or normal IgM. (Yazdani et al., 2019) The disorder is caused by genetic mutations in most cases, although acquired disease related to nephrotic syndrome, infections and neoplasia have been reported. (de la Morena, 2016) Patients are at increased risk for infection, neoplasia and autoimmune disorders, (Leven et al., 2016) and common causes of death include hepatopathy, pneumonia or cancer. There is report of a patient with HlgM suffering autoimmune retinopathy and visual disturbance. (Schuster et al., 2005) Although a tentative link to genetic variants in major histocompatibility complexes have been described in canine SARDS, the variants described did not consistently stratify with phenotype, and the authors conclude that additional genetic or environmental factors are likely involved. (Stromberg et al., 2019) However, until further clarification of our finding occurs, the potential risk of systemic immunosuppressive medications such as prednisone further reducing IgG levels, (Rinkardt et al., 1999) combined with owners’ lack of perceived efficacy of corticosteroid use (Stuckey et al., 2013) suggest that systemic corticosteroids may not be a first-line recommended treatment for dogs with SARDS. An important next step in understanding the pathophysiology of canine SARDS will be to determine if IgM class switch recombination occurs later in disease, or whether there is persistence of the lower IgG and higher IgM levels we detected in early disease.
1.5 Acknowledgements

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1.6 Declarations of interest: None
1.7 REFERENCES


Figure 1. Retinal indirect immunofluorescence: IgG. Representative examples of the staining pattern using serum from dogs with SARDS showing a distinct band of staining in the photoreceptor outer segments (arrowhead; A), which was not present in controls (B; arrowhead delineates approximate location of outer segments) or no serum controls (not shown). Green staining delineates immunofluorescence, blue staining labels nuclei (4′,6-Diamidino-2-Phenylindole; DAPI). All slides had background staining outlining the superficial retinal blood vessels (asterisks in A and B), most likely due to secondary antibody staining of endogenous vascular or intravascular IgG in the retinal section. There was significantly higher staining intensity in the outer segments using serum from dogs with SARDS compared with controls when images were analysed by grader 1 (C). Grader 2 did not achieve significance at the individual retinal layer level (D), but grader 3 had similar findings to grader 1 (E). Scale bar = 20 µm, original magnification x 40. Labels of retinal layers: VIT (vitreous), NFL (nerve fiber layer), GCL (ganglion cell layer), IPL (inner plexiform layer), INL (inner nuclear layer), OPL (outer plexiform layer), IS (photoreceptor inner segments), OS (photoreceptor outer segments), RPE (retinal pigmented epithelium). * P < 0.05, ** P < 0.01 Two-way ANOVA with Bonferroni post-test.
Figure 2. Retinal indirect immunofluorescence: IgM. Representative examples of staining are shown (A,B). Using serum from dogs with SARDS, there was an overall increased staining throughout the retinal layers (A), which was not present in matched controls (B). Green staining is immunofluorescence, blue staining is labelling nuclei (4',6-Diamidino-2-Phenylindole; DAPI). Quantification of retinal images by grader 1 (C) or grader 2 (D) did not identify individual layers with significant differences between dogs with SARDS and controls although for the majority of layers, staining intensity using serum from dogs with SARDS was higher than controls. Scale bar = 20 µm, original magnification x 40. Labels of retinal layers: VIT (vitreous), NFL (nerve fiber layer), GCL (ganglion cell layer), IPL (inner plexiform layer), INL (inner nuclear layer), OPL (outer plexiform layer), IS (photoreceptor inner segments), OS (photoreceptor outer segments), RPE (retinal pigmented epithelium).
Figure 3. Retinal indirect immunofluorescence: IgA and IgE. Representative examples of staining are shown for IgA (A,B) and IgE (E,F). There was no difference in staining for IgA between sera from dogs with SARDS (A), compared with matched healthy controls (B). Neither grader 1 (C) or grader 2 (D) identified any differences in individual retinal layer staining intensity. There was no difference in staining for IgE between sera from dogs with SARDS (E), compared with matched healthy controls (F). Neither grader 1 (G) or grader 2 (H) identified any differences in individual retinal layer staining intensity. Scale bar = 20 µm, original magnification x 40. Green staining is immunofluorescence, blue staining is labelling nuclei (4',6-Diamidino-2-Phenylindole; DAPI). Labels of retinal layers: VIT (vitreous), NFL (nerve fiber layer), GCL (ganglion cell layer), IPL (inner plexiform layer), INL (inner nuclear layer), OPL (outer plexiform layer), IS (photoreceptor inner segments), OS (photoreceptor outer segments), RPE (retinal pigmented epithelium).
Figure 4: Quantification of IgG and IgM using radial immunodiffusion. Serum IgG quantification did not identify any differences between dogs with SARDS and matched healthy controls (A). Serum IgM was significantly higher in dogs with SARDS compared with matched healthy controls (B). Serum total protein was significantly higher in dogs with SARDS compared with matched healthy controls (C). When accounting for this increase in protein concentration by calculating the Ig concentration as a proportion of serum total protein, IgG was significantly lower in the serum of dogs with SARDS compared with healthy controls (D) and IgM remained significantly higher in dogs with SARDS compared with healthy controls (E). There was a significant negative correlation between IgG and IgM in dogs with SARDS, but not in healthy controls (F). Paired student’s t-test (A-E), linear regression (F) * P < 0.05, ** P < 0.01, *** P < 0.001
Supplementary Figure 1: Verification of staining specificity and optimization of indirect immunofluorescence protocol. Positive control tissues were collected post-mortem from a normal dog. Images from tissues fixed in 2% paraformaldehyde are shown. Incubation of anti-IgG (A) and anti-IgM (B) antibodies with submandibular lymph node demonstrated specific plasma cell staining. Incubation of anti-IgA antibody (C) with conjunctiva demonstrated specific subconjunctival plasma cell staining. Incubation of anti-IgE antibody (D) demonstrated specific mast cell staining surrounding cutaneous hair follicles. Indirect immunofluorescence protocol was optimized with pemphigus foliaceus canine patient serum verified to contain anti-epidermal IgG. Although background staining of the dermis was identified in sections incubated without patient serum (E), specific epidermal staining was only identified in sections incubated with patient serum (F). In E and F, the basal epidermis layer is delineated with a vertical white line. PF: pemphigus foliaceous. Scale bar = 20 µm, original magnification x 40.
## 1.9 TABLES

<table>
<thead>
<tr>
<th>Breed</th>
<th>SARDS</th>
<th>Age- and breed-matched healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dachshund (5), Pug (2), Jack Russel terrier (2), Bichon Frise (1), Miniature Schnauzer (1), German Shepherd mixed breed (1), Labrador mixed breed (1), terrier mix (1)</td>
<td>Dachshund (5), Pug (2), Jack Russel terrier (2), Bichon Frise (1), Miniature Schnauzer (1), German Shepherd mixed breed (1), Labrador mixed breed (1), terrier mix (1)</td>
</tr>
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<td>Female spayed (8)</td>
</tr>
<tr>
<td></td>
<td>Female intact (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male neutered (6)</td>
<td>Male neutered (6)</td>
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<tr>
<td>Age (years)</td>
<td>7.98 ± 0.46</td>
<td>8.94 ± 0.85</td>
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<td>Median time since vision loss at recruitment (days)</td>
<td>18 (range 3-29)</td>
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<td>Dark-adapted (20 minutes of dark adaptation) ERG b-wave amplitude at 3cd/m² (µV)</td>
<td>0</td>
<td>190.6 ± 14.1</td>
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<td>Body weight (kg)</td>
<td>11.9 ± 2.3</td>
<td>10.9 ± 2.5</td>
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<tr>
<td>Body condition score (/9)</td>
<td>6.8 ± 0.4 **</td>
<td>4.5 ± 0.2</td>
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<td>Serum total protein (g/dL)</td>
<td>6.7 ± 0.2 ***</td>
<td>5.9 ± 0.1</td>
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<tr>
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<td>5.2-7.3</td>
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<td>Serum albumin (g/dL)</td>
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<td>3.5 ± 0.1</td>
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<td>3.3-3.9</td>
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<td>Serum globulin (g/dL)</td>
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<td>1.7-3.8</td>
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<td>Albumin/globulin ratio</td>
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<td>1.48 ± 0.04</td>
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<td>Plasma protein (g/dL)</td>
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<td>Hematocrit (%)</td>
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<td>(x10^3/µl)</td>
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<td>Band neutrophils (x10^3/µl)</td>
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<td>Upper Limit</td>
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<td>Lymphocytes</td>
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<td>Basophils</td>
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Table 1. Demographics of participants (SARDS and matched healthy control dogs). Mean ± SEM. Paired 2-tailed student’s t-test nonsignificant unless marked: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NA: not applicable.
<table>
<thead>
<tr>
<th>Ig subclass</th>
<th>Category of analysis</th>
<th>Grader 1</th>
<th>Grader 2</th>
<th>Grader 3</th>
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<td>10/14</td>
<td>11/14</td>
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<td>( P &lt; 0.0001 ), 30.1% total variation, ( F = 13.1 )</td>
<td>( P &lt; 0.0001 ), 26.4% total variation, ( F = 10.9 )</td>
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<td>( P = 0.24 ), 0.4% total variation, ( F = 1.4 )</td>
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<td>( P = 0.22 ), 0.4% total variation, ( F = 1.5 )</td>
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<td>Bonferroni post-test</td>
<td>Photoreceptor outer segments: SARDS 0.98 ± 0.22, controls 0.20 ± 0.15, ( P &lt; 0.01 )</td>
<td>Photoreceptor outer segments SARDS 0.73 ± 0.16, controls 0.30 ± 0.04, ( P &gt; 0.05 )</td>
<td>Photoreceptor outer segments: SARDS 0.89 ± 0.21, controls 0.30 ± 0.18, ( P &lt; 0.05 )</td>
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<td>IgM</td>
<td>Proportion of overall grading higher in SARDS</td>
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<td>12/14</td>
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<td>IgA</td>
<td>2-way ANOVA, retinal location</td>
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<td>IgE</td>
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Table 2. Statistical analysis of retinal immunofluorescence assays using 2-way ANOVA. NS: not significant, NA: not applicable.