

Multivariable analysis of the influence of cross-reactive carbohydrate determinant inhibition and other factors on intradermal and serological allergen test results: a prospective, multicentre study

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Background – Serological allergen testing (SAT) is used widely to formulate allergen-specific immunotherapy for atopic dogs. Serum immunoglobulin (Ig)E specific for cross-reactive carbohydrate determinants (CCD) can produce false-positive reactions, creating discrepancy between SAT and intradermal allergen test (IDAT) results.

Objectives – The primary objective was to determine if inhibition of anti-CCD IgE in a commercial assay improved correlation with IDAT. The secondary objective was to assess the influence of dog- and clinic-specific factors, environmental factors, putative allergen exposure and prior medications on intradermal and SAT reactivity.

Animals – Two-hundred and eleven client-owned dogs were enrolled from eight North American dermatology specialty practices.

Methods and materials – Collection of serum samples and IDAT were performed on the same day. Sera were assayed for detection of IgE specific to 25 allergens, before and after treatment with a proprietary inhibitor of anti-CCD IgE. Data for each dog were collected via a questionnaire filled out by veterinary personnel.

Results – The correlation between the testing modalities was fair before (Spearman's rho, $\rho = 0.2092$) and after ($\rho = 0.3042$) inhibition of anti-CCD IgE. Ciclosporin dose ($P = 0.003$), independent of duration of use, and duration of lokivetmab use ($P = 0.001$), independent of dose administered, were associated with statistically significant decreases in IgE concentrations across all allergen types.

Conclusions and clinical relevance – Contrary to previous reports, this study demonstrated unchanged correlation between SAT and IDAT after inhibition of anti-CCD IgE. Ciclosporin dose and lokivetmab treatment duration may have unexplored effects on IgE concentration during SAT.

Introduction

Canine atopic dermatitis (cAD) is a common, genetically predisposed, inflammatory and pruritic skin disease that is most commonly associated with immunoglobulin (Ig)E directed at environmental allergens.^{1,2} Once cAD is diagnosed, allergen testing is recommended as a means to avoid certain allergens and to formulate allergen-specific immunotherapy (ASIT).³ Both intradermal allergen testing (IDAT) and *in vitro* serology for canine allergen-specific IgE are used widely in clinical veterinary practice.

Previous studies have demonstrated discrepancies between IDAT and serum allergen testing (SAT) which can lead to the errant inclusion of irrelevant allergens in a subject's ASIT.^{4,5} These inconsistencies may be due partially to false-positive serological reactions generated by cross-reactive carbohydrate determinants (CCD).^{5,6} CCDs are unique, highly antigenic carbohydrate structures found on plant and insect allergens.⁷ Previous studies of human patients have identified these structures as a source of cross-reactivity and polysensitisation in serological testing despite having limited clinical relevance.^{8–10} In veterinary studies, the prevalence of anti-CCD IgE in dogs has been reported to be similar to that which occurs in people.^{7,11,12} In human and canine sera, the presence of anti-CCD activity frequently confers polysensitisation to plant pollens on SAT.^{6,12,13}

Inhibition of an individual's anti-CCD IgE might result in more reliable serum testing by reducing false-positive reactivities and subsequent erroneous diagnosis of

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polysensitisation. This was first demonstrated in human medicine after applying a CCD blocker prepared from pineapple stem bromelain and human serum albumin.⁶ A recent study of 31 dogs with cAD showed enhanced agreement between serum and intradermal testing after blocking anti-CCD IgE in sera with a propriety solution.¹²

The primary objective of the present study was to determine if inhibition of anti-CCD IgE improved correlation with IDAT results for dogs in which intradermal testing and serum collection were performed simultaneously.¹⁴ We hypothesised that a modest change in correlation would be achieved. The secondary objective was to assess the influence of dog- and clinic-specific factors, environmental factors, putative allergen exposure history and prior medication use on intradermal and enzyme-linked immunosorbent assay (ELISA) test reactivity. We hypothesised that the season of testing, differences in climate, concentration of allergens used for IDAT, and the prior use of antipruritic drugs would influence test results in a multivariable statistical model.

Methods and materials

This prospective study was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania School of Veterinary Medicine. Informed consent was obtained from pet owners before each subject's enrollment. Two-hundred and eleven client-owned dogs with cAD presented to eight North American dermatology/allergy referral clinics were included in the study. The clinics were located in the following metropolitan areas of the USA: Philadelphia, PA and Washington, DC (mid-Atlantic region); Boston, MA (northeast); Orlando, FL (southeast); and Houston and Austin, TX (south central/Gulf Coast).

Subject inclusion criteria

Subjects were included once a clinical diagnosis of cAD was made based on compatible history and clinical signs, according to the criteria of Favrot.¹⁵ Specific exclusion criteria were not required for dogs with potential concurrent hypersensitivities (e.g. adverse food reaction, flea bite allergy). Injectable, oral and topical steroids were withdrawn within 90 days, 30 days and 14 days, respectively, before IDAT. Oral or topical antihistamines and oral essential fatty acids were withdrawn 14 days before IDAT. A minimum body weight of 8.5 kg was required in order to safely collect the volume of blood for subsequent SAT.

Subject questionnaire

Signalment data collected for all subjects included age, breed, sex, body weight, housing status (predominantly indoors, outdoors or mixed) and cohabitation with a cat. Each dog's historical pattern of pruritus was documented, including the duration and seasonality of clinical signs (seasonal, perennial, or perennial with seasonal exacerbation) and season(s) of greatest clinical sign severity. Recent exposures to parasites (presence of fleas, *Sarcoptes scabiei* and/or intestinal parasites) within the past three months and current use of flea, tick and heartworm prevention were documented. The presence of *Malassezia* infection (skin and/or ear canal) within the 30 days before IDAT was noted as were any histories of known adverse food reactions and subsequent elimination diet trials (including novel or hydrolysed protein products). Data were collected to document the dose and duration of antipruritic drugs used immediately before IDAT, including oclacitinib (Apoquel, Zoetis; Kalamazoo, MI, USA), lokivetmab (Cytoint; Zoetis) and ciclosporin (Atopica, Elanco; Greenfield, IN, USA), and use of ASIT within six months of testing. The

questionnaire completed by participating veterinary personnel is presented in Table S1 in Supporting information.

Intradermal testing

Participating clinics used their own routine, region-specific panels of allergens for IDAT. Intradermal reactivity to 25 allergens used in common by all participating clinics (Table 1) were evaluated by objective and subjective criteria. The concentrations used for IDAT varied between participating veterinary hospitals and between allergen classes. Histamine phosphate and 0.9% saline served as positive and negative controls, respectively. If necessary, the dogs were sedated with dexmedetomidine (Dexdomitor, Zoetis) intravenously, using a dose approximating 5 µg/kg body weight. Subjective evaluations of IDAT reactions were based on erythema, wheal size, turgidity and slope of each reaction ranging from 0 (=negative) to 4 (=high reactivity) as reported previously.² Each reaction diameter was assessed as the average of the length and width in millimetres, and this value was then multiplied by firmness and erythema scores, each graded on a scale of 1–3, to determine an objective "global wheal score" as described previously.^{16–17} The global wheal score for each individual allergen was compared to the mean of the wheal score values for the positive and negative controls for that dog. If an allergen's global wheal score exceeded the control threshold, it was declared to be positive.

Serum testing

For each participant, serum harvested from 20 mL of whole blood, which was collected at the time of IDAT, was shipped by 2nd-day carrier to Stallergenes Greer Laboratories (Lenoir, NC). An aliquant of each serum sample was evaluated immediately and a serological testing report was provided to the submitting veterinarian. The remaining volume of each sample was stored at –80°C. At the conclusion of the study, these stored samples were evaluated, with and

Table 1. Allergens tested with both intradermal allergen test (IDAT) and serological allergen testing (SAT)

Mites	
<i>Dermatophagoides farinae</i>	House dust mite
<i>Dermatophagoides pteronyssinus</i>	House dust mite
<i>Tyrophagus putrescentiae</i>	Food/storage mite
Insects	
<i>Ctenocephalides felis</i>	Flea saliva
Culicidae	Mosquito
Danders	
<i>Felis catus</i> (domesticus)	Cat dander
Moulds and Fungi	
<i>Alternaria alternata</i>	
<i>Cladosporium herarum</i>	
<i>Penicillium notatum</i>	
<i>Malassezia pachydermatis</i>	
Trees	
<i>Salix nigra</i>	Black willow
<i>Morus rubra</i>	Red mulberry
<i>Quercus rubra</i> , <i>Q. velutina</i> , <i>Q. alba</i>	Oak mix
<i>Pinus strobus</i> , <i>P. echinata</i> , <i>P. taeda</i>	Pine mix
<i>Juniperus virginiana</i>	Red cedar
Grasses	
<i>Lolium perenne</i>	Perennial ryegrass
<i>Phleum pratense</i>	Timothy grass
<i>Sorghum halepense</i>	Johnson grass
<i>Cynodon dactylon</i>	Bermuda grass
Weeds	
<i>Xanthium strumarium</i>	Cocklebur
<i>Rumex crispus/R. acetosella</i>	Dock/sorrel mix
<i>Plantago lanceolata</i>	English plantain
<i>Chenopodium album</i>	Lamb's quarter
<i>Amaranthus hybridus</i> , <i>A. palmeri</i> , <i>A. retroflexus</i>	Pigweed mix
<i>Ambrosia trifida</i> , <i>A. artemisiifolia</i>	Ragweed mix

without CCD inhibitor, over a five day period using procedures defined previously.¹⁴

Following incubation of allergen-coated wells with an appropriately diluted serum sample, allergen-specific IgE was detected using a secondary antibody mixture of biotinylated monoclonal anti-IgE antibodies, streptavidin alkaline phosphatase as the enzyme conjugate and *p*-nitrophenylphosphate (pNPP) as substrate reagent. Specific IgE reactivity to the allergens then was estimated by determining the absorbance of each well measured at 405 nm using an automated plate reader.

All results were expressed as ELISA absorbance units (EAU) which are background-corrected observed responses expressed as milli absorbance.¹⁴ The buffers used throughout included: (a) well-coating buffer: 0.05 M sodium carbonate bicarbonate buffer, pH 9.6; (b) wash buffer: phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween 20 and 0.05% sodium azide; and (c) serum and reagent diluent buffer: PBS, pH 7.4, containing 1% fish gelatin, 0.05% Tween 20 and 0.05% Proclin. For inhibition evaluations, the bromelain CCD-inhibitor (BROM-CCD) was added to the diluent buffer at the defined concentration of 2.5 mg/mL before addition of serum sample.

The BROM-CCD, which contains the carbohydrate components present in bromelain, was prepared in-house and remains a proprietary product of Stallergenes Greer.

Statistical analysis

All analyses were conducted with STATA 15MP (StataCorp; State College, TX, USA) with two-sided tests of hypotheses and $P < 0.05$ as the criterion for statistical significance. Descriptive analyses included computation of means [with 95% confidence intervals (CI)], standard deviations, medians, interquartile ranges (IQR) of continuous variables and tabulation of categorical variables. Tests of normal distribution were performed to determine extent of skewness, and transformation methods (e.g. logarithmic) were used to normalise the distribution of seriously skewed variables. Frequency counts and percentages were used for categorical variables (e.g. sex, signalment).

Similar to a recent study, a dog was considered to be polysensitised when the majority of reactions in a specific subgroup (at least two of the three mites, three of four moulds and fungi, three of four grasses, three of five trees and four of six weeds) were positive.¹² The number of allergens in the groups "danders" ($n = 1$) and "insects" ($n = 2$) measured in both tests were too low for a majority to be claimed, so these were not included in evaluations of polysensitisation.

Agreement between IDAT reactivity (both subjective and wheal scores) and allergen-specific IgE reactivity (both before and after absorption of CCD antibodies) was estimated with Spearman's rank correlation coefficient (ρ , p). Correlations between subjective and objective (wheal score) values for IDAT also were estimated. Correlation coefficients were interpreted in accordance with Cohen's kappa with the following values: <0 no agreement, 0–0.20 slight agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial agreement and 0.81–1 almost perfect agreement.¹⁸

Inference statistical analysis was conducted in three steps. First, univariate analysis was used to identify statistically significant confounders ($P < 0.2$) associated with the outcomes of interest. Secondly, in order to identify a subset of significant predictors associated with the outcome of interest, lasso regression was performed. Third and finally, multivariable regression models were constructed to test associations between outcomes measures (IDAT and serological reactivity) and many subject- and clinic-level variables. Fixed effects on the subject-level included signalment data, clinical parameters, putative environmental exposures and prior drug therapies. Clinic-level fixed effects included latitude of clinic location, allergen concentration used during IDAT and variability in clinic IDAT interpretation. Robust estimation of the variance was used to permit for small departures from normality. Least significant difference adjustment for multiple comparisons was used.

Results

A total of 211 dogs were enrolled, including 73 females (72 spayed) and 136 males (127 neutered). For two dogs, sex was not reported. The mean age at time of inclusion was 3.75 years (range 1–11 years), and the mean duration of clinical signs was 25.55 months (range 0.23 to 111.85 months). Age and duration of clinical signs were not reported for two and nine dogs, respectively.

Twenty-one of 211 dogs (9.95%) were actively receiving or had recently received ciclosporin for a median duration of 4.27 weeks (range 1.14–59 weeks), and the median daily dose was 4.81 mg/kg (range 4.19–7.72 mg/kg). For dogs receiving concurrent ketoconazole, the ciclosporin dose reported by the attending veterinarian was doubled for analytical purposes.¹⁹ Ninety-two of 211 dogs (43.60%) were actively or had recently received daily oclacitinib for a median duration of 15 weeks (range 1.14–156 weeks) and the median dose was 0.52 mg/kg (range 0.33–1.16 mg/kg). Forty of 211 dogs (18.96%) had received lokivetmab before testing for a median duration of four weeks (1–104 weeks) with a median dose of 2.37 mg/kg (range 1.63–3.25 mg/kg), although mean frequency of administration was not known. Two dogs were already receiving ASIT at the time of enrollment, one for 13 weeks and other for 52 weeks.

Agreement between tests

The Spearman's ρ correlation coefficients for agreement between objective and subjective IDAT scores and the corresponding SAT test IgE results, both before and after inhibition by BROM-CCD, are summarised in Table 2. Objective and subjective IDAT results were analysed both as continuous variables and as binary variables indicating either no reaction or a positive reaction. In the total subject population, there were >5,000 results evaluated. There was substantial agreement ($\rho = 0.6137$) between objective and subjective IDAT results. There was fair agreement ($\rho = 0.2972$, $\rho = 0.3042$) between objective IDAT results and serological IgE before and after inhibition of anti-CCD IgE, respectively.

Serum IgE results

Serum test results were evaluated for the presence of polysensitisation, both before and after anti-CCD IgE inhibition by BROM-CCD. These results are illustrated in Table 3. Before inhibition of anti-CCD IgE by BROM-CCD, all subgroups had apparent polysensitisation ranging from 14.69% to 55.45%. Once treated with BROM-CCD, there was equivocal change in percentage of patients expressing polysensitisation to mites, while there was 54.05%, 53.62% and 46.00% reduction in rates for trees, grasses and weeds, respectively.

From the sampled population, 12.80% (27 of 211) of dogs were defined to be nonreactive (EAU <150) to all of the allergens tested both pre- and postinhibition by BROM-CCD while the remaining population had at least one positive (EAU >150) reaction. Thirty-two of 211 (15.17%) dogs had an increased number of positive reactions following inhibition relative to their initial results, and the mean increase in positivity was 1.68 allergens. One hundred and nine of 211 (51.66%) dogs' reactivities

Table 2. Spearman's rho (ρ) agreement for objective and subjective intradermal allergy test (IDAT) results and serum allergy test IgE results, pre- and post-inhibition*

	Objective IDAT	Subjective IDAT	IgE pre-inhibition	IgE post-inhibition
Objective IDAT	1.000			
Subjective IDAT	0.6137 ($P < 0.001$)	1.000		
IgE Pre-Inhibition	0.2972 ($P < 0.001$)	0.2543 ($P < 0.001$)	1.000	
IgE Post-Inhibition	0.3042 ($P < 0.001$)	0.2550 ($P < 0.001$)	0.8492 ($P < 0.001$)	1.000

*Values < 0 indicate no agreement, 0–0.20 slight, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 substantial and 0.81–1 almost perfect agreement.

Table 3. Polysensitization with serum testing for allergen-specific IgE of atopic dogs, pre- and post-inhibition of anti-cross carbohydrate determinant IgE*

Allergen Subgroup	Pre N	%Total	Post N	%Total	% Reduction
Mite	117.0	55.45	120.0	56.87	–2.56
Moulds	31.0	14.69	24.0	11.37	22.58
Trees	37.0	17.54	17.0	8.06	54.05
Grasses	69.0	32.70	32.0	15.17	53.62
Weeds	50.0	23.70	27.0	12.80	46.00

*If the majority of reactions within an allergy subgroup were positive (>150 EAU, at least 2 of three mites, three of four molds, three of five trees, three of four grasses, and four of six weeds), the dog was considered to be polysensitized for this particular subgroup.

decreased following inhibition with a mean decrease of 4.56 allergens.

For allergens considered positive on serological testing, the EAU value after BROM-CCD inhibition was evaluated and compared against the initial result to determine the inhibition response. Within the mites subgroup, only 40 of 323 (12.38%) positive reactions exhibited a moderate ($>40\%$) or greater inhibition response. However, 136 of 204 (66.67%) of positive tree pollens, 196 of 330 (59.40%) of positive grass pollens and 154 of 339 (45.43%) of positive weed pollens demonstrated at least moderate ($>40\%$) inhibition. These subgroups had near complete inhibition ($>80\%$) of 32 of 204 (15.69%), 43 of 330 (13.03%) and 41 of 339 (12.09%) positive reactions, respectively. These results are summarised in Table 4.

After near complete inhibition of reactions within these subgroups, 17 of 211 (8.06%) dogs were polysensitized to mites only after having been previously considered polysensitized to a majority (at least three of five) of the allergen subgroups. There were seven of 211 (3.32%) dogs that were considered polysensitized to the majority of nonmite subgroups (three of four) before inhibition that no longer qualified as such after BROM-CCD addition.

Multivariable regression analysis

Multivariable regression analysis was performed to determine if any subject-, clinic- or allergen-level fixed effects were significantly associated with objective IDAT or SAT results. When adjusted for allergen type and latitude, dogs were significantly more likely to react to the house dust mite (HDM) and storage mite group on IDAT ($P = 0.005$) when compared to fungi (i.e. moulds and *Malassezia*). They were significantly less likely to react to cat dander ($P < 0.001$) and the insect group (i.e. flea and mosquito; $P = 0.031$). When adjusted for allergen testing concentration, ciclosporin dose, duration of lokivetmab therapy, and latitude, similar associations were noted to the SAT results, where reactivity to “mites” ($P < 0.001$)

was significantly more likely, and reactivity to cat dander ($P < 0.001$) and “insects” ($P < 0.001$) were significantly less likely than reactivity to the fungi. There were no significant differences in reactivity between the fungi and pollen groups with either testing modality.

When adjusted for allergen testing concentration, allergen type, ciclosporin dose, duration of lokivetmab therapy, and latitude, the dose of ciclosporin used before testing was associated with a significant ($P = 0.003$) negative effect on mean SAT reactivity. However, the duration of use – as an independent variable – was not. For each 1 mg/kg increase in dose, the mean EAU decreased by 21.36 units. In the same analysis, the duration of lokivetmab use before testing also was associated with a significant ($P = 0.001$) negative effect on serological IgE results. For each week of increased use, the mean EAU decreased by 2.69 units. There were no statistical associations between the magnitude of IDAT reactions and use of antipruritic drugs before testing. However, when adjusted for SAT results, allergen testing concentration, latitude, clinic and allergen type, the magnitudes of IDAT results reported by two clinics in the mid-Atlantic region and one clinic in the south-central region were significantly greater ($P \leq 0.011$) than the other clinics. This result was independent of all other variables (i.e. this effect was not associated with the geographical latitudes of the practice locations nor with the concentrations of allergens used for IDAT). The results of the multivariable regression analysis for objective IDAT and SAT results are summarised in Table 5.

Discussion

This multi-institutional study sought to investigate how inhibition of IgE antibodies specific for CCDs in serum samples affects the correlation between serum ELISA and IDAT results. There was no significant change in the correlation coefficients between the two testing

Table 4. Inhibition of allergen-reactive sera in a population of dog sera samples after inhibition of anti-cross carbohydrate determinant (CCD) IgE by the bromelain CCD-inhibitor

% Inhibition	Mites		Moulds		Trees		Grasses		Weeds	
	N	%	N	%	N	%	N	%	N	%
>80%	1	0.309598	5	2.392344	32	15.68627	43	13.0303	41	12.0944
60–80%	6	1.857585	9	4.30622	67	32.84314	72	21.81818	49	14.45428
40–60%	12	3.71517	25	11.96172	37	18.13725	81	24.54545	64	18.87906
20–40%	21	6.501548	63	30.14354	35	17.15686	69	20.90909	63	18.58407
<20%	64	19.81424	60	28.70813	20	9.803922	40	12.12121	51	15.04425
≤0%	219	67.80186	47	22.48804	15	7.352941	25	7.575758	64	18.87906
Positive (>150)	323	100	209	100	204	100	330	100	339	100

Table 5. Factors with significant effects on post-inhibition IgE results and objective IDAT results identified by the final multivariable models. All other clinic- and subject-level variables were non-significant

	Variable	Coefficient	Standard error	P	LCL	UCL
Post-Inhibition IgE Results	Danders	−71.21	14.41	<0.001	−111.06	−46.79
	Mites	474.01	60.25	<0.001	355.92	592.1
	Insects	−88.16	20.67	<0.001	−128.68	−47.63
	Cyclosporine dose	−21.36	9.36	0.003	−44.82	−8.13
	Lokivetmab duration	−2.69	1.01	0.001	−4.71	0.73
Objective IDAT Results	South central clinic	91.78	28.36	0.001	36.19	147.37
	Mid-Atlantic clinic 1	155.35	34.03	<0.001	88.65	222.05
	Mid-Atlantic clinic 2	85.79	33.90	0.011	19.35	152.23
	Danders	−43.69	9.29	<0.001	−61.90	−25.47
	Mites	161.04	27.58	0.005	107.00	215.09
	Insects	−25.9	11.97	0.031	−49.37	−2.43

LCL lower confidence level, UCL upper confidence levels.

modalities after CCD treatment, despite apparent neutralisation of polysensitised individuals. These results contrast with those observed in a recent study of a much smaller population of dogs despite similarities in the detection of polysensitisation to pollens in unblocked sera.

Because the BROM-CCD inhibitor eliminates only the reactivity of the CCD portion of the allergen, the unchanged correlation of the two test modalities is not surprising. Although a substantial reduction in the IgE signal magnitude was evident following the addition of BROM-CCD, allergen-specific IgE reactivity remained to most of the allergens tested. Combined with the presumption that anti-CCD IgE are not involved in IDAT reactivity, the correlation of results before and after BROM-CCD may not differ. The true utility of CCD inhibition during SAT is a more accurate determination of allergens selected for an immunotherapeutic regimen, and minimising the confusion created by polysensitisation is valuable to clinicians and clients alike.

False-positive reactions due to anti-CCD IgE can create discrepancies between SAT and IDAT results, and could lead to the inclusion of irrelevant allergens in ASIT formulation.^{20,21} In this study, a propriety preparation containing the carbohydrate components found in bromelain, a protease present in the fruit and stems of pineapples, was added to the ELISA to inhibit sera anti-CCD IgE as described in a previous report.¹⁴ The addition of BROM-CCD generally reduced EAU results but positive reactions above the >150 EAU threshold were still maintained in most dogs.

The tendency for polysensitisation to be neutralised after CCD inhibition was assessed for each allergen type. After addition of BROM-CCD, all allergen subgroups were at least partially inhibited with the exception of mites. For mites, polysensitisation infrequently resolved, similar to human data where blocking anti-CCD IgE rarely affects positive mite reactions. This lack of change is likely because arthropods contain few or no CCDs.^{8,22} The pollen allergens demonstrated the most substantial inhibition, in line with previous results.^{12,14} Weed, tree and grass pollens share similar carbohydrate structural elements which can react with circulating anti-CCD IgE, although these reactions cannot stimulate mast cell degranulation and do not invoke relevant clinical signs.²³

In the present study, there was no specific detection method utilised to quantify the number of dogs carrying anti-CCD IgE. This population was conservatively estimated on the basis of dogs initially rated as polysensitised to the majority of allergen types (at least three of five) before inhibition with subsequent neutralisation of two or more of these subgroups. Seventeen of 211 (8.06%) dogs were polysensitised to a majority of subgroups (three of five mites, fungi, grasses, tree and/or weeds) pre-inhibition and were polysensitised only to mites postinhibition. Another seven dogs (3.32%) were polysensitised to a majority of subgroups, excluding mites, and were polysensitised to one or zero subgroups after BROM-CCD addition. Together, this represents only twenty-four of 211 (11.37%) dogs for which there was a strong indication of a role for anti-CCD IgE. There may be additional carriers of anti-CCD IgE within the study

population not counted by these means. In previous studies, the presence of anti-CCD IgE was detected in 38.7% and 24% of dogs, similar to the known incidence in humans.^{11,12} More studies are needed to demonstrate the true incidence of these antibodies in both atopic and nonatopic patients. Furthermore, if the study population is truly deficient in carriers, a higher incidence may have allowed for improved correlation between testing modalities following inhibition.

There are limited studies investigating the effect of the timing of when SAT is performed relative to a dog's reported seasonality of clinical signs. Two previous reports suggest that allergen-specific IgE fluctuate seasonally whereas another indicated an increase in positive reactivity 60 days after the conclusion of the pollen season.^{24–26} These investigations were very brief in duration and included only small numbers of atopic dogs. In the present study, there were no statistical differences in rates of positive reactivity to any of the allergen groups, as defined by SAT or IDAT, according to either the season during which testing occurred or the reported seasonality of the dogs' clinical signs. However, the dogs did not undergo serial testing during different seasons, so the multivariable analysis can provide only circumstantial evidence for lack of a seasonal influence on test results.

A statistically significant negative association between ciclosporin dose and SAT results was identified by the multivariable analysis. For each 1 mg/kg dose increase of ciclosporin, there was a 26.5 unit decline in ELISA EAU values across all allergen types, independent of the duration of ciclosporin administration. Because an IgE concentration and its respective EAU output does not relate linearly, the dose-related effect of ciclosporin would be even more pronounced if expressed as an IgE concentration.¹⁴ There was no apparent effect of ciclosporin on IDAT reactivity. Three previous studies which reported on eight dogs with experimentally induced flea-allergic dermatitis, 16 dogs with cAD and four dogs sensitised to *Ascaris suum*, respectively, showed that ciclosporin administered at the standard once-daily dose (5 mg/kg) had no significant effect on serum IgE levels.^{27–29} The higher statistical power of the present study as well as a wider dose range of ciclosporin used across our study population may explain the significance reported here. Ciclosporin is a highly specific inhibitor of T-cell activation and may affect production of allergen-specific IgE by plasma cells through downstream effects on cytokine signalling by T_H2 cells.³⁰ Further studies are needed to explore the effect of ciclosporin on serum allergen-specific IgE and whether IDAT would be the preferred testing method for atopic dogs receiving this medication.

The duration (in weeks) of lokivetmab administration also was associated with a statistically significant, yet minor, decrease in serum ELISA EAUs. For each additional week of lokivetmab use, there was a 2.7 unit decrease in EAU across all allergen types. This effect was independent of lokivetmab dose. This compound is still relatively novel, and only two prior studies have investigated its effects on allergen-specific IgE. In these studies, the IgE concentrations for eight beagles sensitised to *D. farinae* and one dog with spontaneous cAD were not apparently affected, although in neither population had

any individual dog received lokivetmab for longer than seven weeks.^{31,32} The present study included a much larger population of dogs, some of which had been receiving lokivetmab for two years, and tested a wider array of allergens. It is not clear how lokivetmab's effects on interleukin (IL)-31 may directly or indirectly influence allergen-specific IgE; neither dose nor duration of lokivetmab use affected IDAT results. Oclacitinib also affects IL-31 but was not found to have any significant associations with IDAT or SAT results.³³ While both ciclosporin dose and lokivetmab duration may affect SAT IgE concentration, the clinical relevancy of this finding may be limited. If a particular allergen's EAU otherwise would be greater than the positive threshold of ≥ 150 , then this value could be interpreted as negative owing to medication use. However, in most instances, positive reactivity may only be slightly reduced where EAU values are substantially higher than the clearance threshold, and SAT may still be recommended for most patients receiving either medication. Atopic dogs receiving high-dose ciclosporin or long-term lokivetmab could be more likely to have dampened reactivity on SAT.

Some participating veterinary clinics were significantly more likely to generate positive IDAT global wheal scores than others. Global wheal scores are not often used in clinical practice but have been used in studies to standardise skin allergen test results. Within the wheal score algorithm, the observer must subjectively assign a 1–3 grade to the firmness and erythema of a reaction, and these grades subsequently are multiplied by the wheal dimensions to generate the mean wheal score. The clinics that produced these higher wheal scores were all single-doctor practices, and their results were compared to clinics with multiple investigators. Because there were no other clinic-level factors to explain this difference, we speculate that the systematic differences in subjective grading may explain the significance of this clinic level variable. Further refinement of this scoring system and the techniques used might be necessary to standardise and validate this approach better.

Previously HDM have been shown to yield the highest rate of positive reactivity among dogs with cAD, regardless of testing method.^{34,35} The results of the present investigation found HDM and storage mites to have significantly higher positive reactivity than other allergen groups. Contrary to prior findings, mosquito, flea, and cat dander were significantly less likely to produce positive IDAT or IgE reactivity than the other allergen types.³⁴ The dogs tested in this study may differ from previously surveyed groups by unknown environmental and/or lifestyle factors which could influence the positivity rate of these allergens. Although the allergen concentrations used for IDAT were not associated with any significant difference in objective IDAT reactivity, there was deviation from recommended allergen testing thresholds for mites, insects, and cat dander. Although the recommended dilution for *D. pteronyssinus* is 100–200 PNU/mL, 176 of 211 (83.41%) of samples were tested at $\geq 1,000$ PNU/mL.¹⁹ All samples were ≥ 180 PNU/mL for *T. putrescentiae*, exceeding the recommended dilution of 75 PNU/mL.³⁶ Exceeding the recommended concentration may have contributed to the high rate of positive reactivity in the

mite subgroup. For cat dander, 80 of 211 (37.91%) samples were tested at the concentration 1,000 PNU/mL while it is recommended to test these allergens with $\geq 1,250$ PNU/mL.³⁶ All participating clinics tested for mosquito reactivity at concentrations below the recommended threshold of 1,750 PNU/mL.³⁶ Failing to use the recommended allergen testing concentrations may be linked to the significantly lower rate of positive reactions to these allergens.

A possible limitation of this study could be the inclusion of a relatively small number of dogs carrying anti-CCD IgE. Theoretically, inclusion of more polysensitised subjects may have led to greater improvement in correlation between the two tests. Likewise, there was a relatively small number of shared allergens ($n = 25$) tested between the participating clinics. Clinical allergen testing commonly uses many more allergens appropriate for the clinic's location. While these allergens are clinically important and relevant across multiple geographical regions, using more extensive allergen panels may have improved our ability to identify anti-CCD IgE-induced polysensitisation.

Other potential limitations include missing data and an uneven enrollment of cases across the various geographical areas. Despite collection of signalment and historical data for each subject, some owners were unaware of their pet's recently used medications and preventatives, seasonality of clinical signs and/or potential exposures. How these missing data may have affected results is unknown.

In summary, the present study failed to demonstrate a clinically relevant change in correlation between IDAT and SAT after inhibition of anti-CCD IgE by BROM-CCD. There was apparent neutralisation of anti-CCD IgE in select dogs and general inhibition observed across all non-HDM and storage mite allergens. Ciclosporin dose, independent of duration, and lokivetmab duration of use, independent of dose, were associated with significantly reduced mean IgE concentrations, although the clinical relevancy of these findings may be limited. Compared to other allergen types, mite allergens were significantly more likely to be positive on IDAT and SAT results, while the insect subgroup and cat dander were significantly less likely to be reactive. Select clinics were associated with significantly higher wheal score results, but a clear reason for disparate results was not identified. Collectively, the results of this study reveal an unprecedented effect of ciclosporin and lokivetmab on SAT results and build upon the existing evidence of the importance of anti-CCD IgE, although future studies are needed to investigate further these findings.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Questionnaire filled out by veterinary personnel for each enrolled subject

Résumé

Contexte – Les tests allergologiques sériques (SAT) sont largement utilisés pour composer l'immunothérapie spécifique d'allergènes des chiens atopiques. Les immunoglobulines (Ig) E sériques spécifiques des CCD (cross-reactive carbohydrate determinants) peuvent engendrer des faux positifs, créant un décalage entre les résultats des intradermoréactions (IDAT) et les SAT.

Objectifs – Le premier objectif était de déterminer si l'inhibition des IgE anti-CCD dans un test commercial, améliorerait la corrélation avec les IDAT. Le deuxième objectif était d'évaluer l'influence des facteurs spécifiques des cliniques et des chiens, des facteurs environnementaux, de l'exposition potentielle aux allergènes et des traitements antérieurs sur la réactivité intradermique et SAT.

Sujets – Deux cent onze chiens de propriétaires ont été inclus de huit services de dermatologie d'Amérique du Nord.

Matériels et méthodes – La collecte des échantillons sériques et IDAT a été réalisée le même jour. Les sera ont été soumis à la détection d'IgE spécifiques de 25 allergènes, avant et après traitement avec un inhibiteur d'IgE anti-CDD. Les données de chaque chien ont été collectées via un questionnaire rempli par le personnel vétérinaire.

Résultats – La corrélation entre les modalités des tests était faible avant (Spearman's rho, $\rho = 0.2092$) et après ($\rho = 0.3042$) inhibition des IgE anti-CCD. La dose de ciclosporine ($P = 0.003$), indépendamment de la durée d'utilisation, et la durée d'utilisation du lokivetmab ($P = 0.001$), indépendamment de la dose administrée, ont été associés à une diminution statistiquement significative des concentrations d'IgE pour tous les types d'allergènes.

Conclusions et importance clinique – Contrairement aux publications antérieures, cette étude montre une corrélation inchangée entre SAT et IDAT après inhibition des IgE anti-CCD. La dose de ciclosporine et la durée du traitement au lokivetmab pourraient voir des effets inexplorés sur les concentrations d'IGE au cours des SAT.

Resumen

Introducción – la prueba serológica de alérgenos (SAT) se usa ampliamente para formular inmunoterapia específica de alérgenos para perros atópicos. La inmunoglobulina (Ig)E sérica específica para determinantes de carbohidratos de reacción cruzada (CCD) puede producir reacciones positivas falsas, creando discrepancias entre los resultados de SAT y la prueba intradérmica (IDAT).

Objetivos – el objetivo principal era determinar si la inhibición de IgE anti-CCD en un ensayo comercial mejoraba la correlación con IDAT. El objetivo secundario fue evaluar la influencia de factores específicos de la clínica y del perro, factores ambientales, exposición supuesta a alérgenos y medicamentos previos sobre la reactividad intradérmica y SAT.

Animales – se inscribieron doscientos once perros de propietarios particulares de ocho clínicas especializadas en dermatología de América del Norte.

Métodos y materiales – La recolección de muestras de suero y la IDAT se realizaron el mismo día. Se analizaron los sueros para la detección de IgE específica para 25 alérgenos, antes y después del tratamiento con un inhibidor patentado de IgE anti-CCD. Los datos de cada perro se recopilaban a través de un cuestionario completado por personal veterinario.

Resultados – la correlación entre las modalidades de prueba fue regular antes (ρ de Spearman, $\rho = 0,2092$) y después ($\rho = 0,3042$) de la inhibición de la IgE anti-CCD. La dosis de ciclosporina ($P = 0,003$), independientemente de la duración del uso y la duración del uso de lokivetmab ($P = 0,001$), independientemente de la dosis administrada, se asociaron con disminuciones estadísticamente significativas en las concentraciones de IgE en todos los tipos de alérgenos.

Conclusiones y relevancia clínica – contrariamente a informes anteriores, este estudio demostró una correlación sin cambios entre SAT e IDAT después de la inhibición de IgE anti-CCD. La dosis de ciclosporina y la duración del tratamiento con lokivetmab pueden tener efectos inexplorados sobre la concentración de IgE durante la SAT.

Zusammenfassung

Hintergrund – Serologische Allergentests (SAT) werden weithin eingesetzt, um eine Allergen-spezifische Immuntherapie für atopische Hunde zu formulieren. Serum Immunglobulin (Ig)E spezifisch für kreuzreaktive Kohlenhydratdeterminanten (CCD) können falsch-positive Reaktionen verursachen, was eine Diskrepanz zwischen SAT und Intradermaltest (IDAT) Ergebnissen verursacht.

Ziele – Das primäre Ziel war es festzustellen, ob die Inhibierung von anti-CCD IgE in einem kommerziellen Assay die Korrelation mit IDAT verbessern würde. Das zweite Ziel war es, den Einfluss auf die Hunde- und Klinik-spezifischen Faktoren, Umweltfaktoren, vermeintliche Allergenexposition und frühere Medikationen auf die intradermale und die SAT Reaktivität zu beurteilen.

Tiere – Zweihundertelf Hunde im Privatbesitz, die Patienten in acht Nordamerikanischen Dermatologie Spezialpraxen waren, wurden in die Studie aufgenommen.

Methoden und Materialien – Es wurden Serumproben genommen und am selben Tag ein IDAT durchgeführt. Die Sera wurden vor und nach der Behandlung mit einem speziellen anti-CCD IgE Inhibitor, auf spezifisches IgE auf 25 Allergene untersucht. Die Daten von jedem Hund wurden mittels Fragebogen, welcher durch das veterinärmedizinische Personal ausgefüllt wurde, erfasst.

Ergebnisse – Die Korrelation zwischen den Testmodalitäten war moderat vor (Spearman's ρ , $\rho = 0,2092$) und nach ($\rho = 0,3042$) Inhibierung von anti-CCD IgE. Die Ciclosporindosis ($P = 0,003$), unabhängig von der Dauer der Anwendung, die Dauer der Lokivetmab Anwendung ($P = 0,001$), unabhängig von der verabreichten Dosis, wurden mit statistisch signifikant niedrigeren IgE Konzentrationen auf alle Allergentypen in Zusammenhang gebracht.

Schlussfolgerungen und klinische Bedeutung – Entgegen den früheren Berichten zeigte diese Studie nach der Inhibierung von anti-CCD IgE eine unveränderte Korrelation zwischen SAT und IDAT. Die Ciclosporindosis und die Lokivetmab Behandlung könnte noch unerforschte Auswirkungen auf die IgE Konzentration während dem SAT haben.

要約

背景 – 血清学的アレルギー検査 (SAT) は、アトピー犬のアレルギー特異的免疫療法の処方に広く用いられている。交差反応性糖鎖決定基 (CCD) に特異的な血清免疫グロブリン (Ig)Eは偽陽性反応を示し、SATや皮内アレルギー検査 (IDAT) の結果に矛盾を生じさせることがある。

目的 – 第一の目的は、市販の検査法で抗CCD IgEを阻害することにより、IDATとの相関性が向上するかどうかを判断することであった。第二の目的は、皮内反応およびSAT反応に対する犬、診療所固有因子、環境因子、推定アレルギー暴露および投薬歴の影響を評価することである。

供試動物 – 北米の8つの皮膚科専門病院から、クライアント所有犬211頭が登録された。

材料と方法 – 血清サンプルの収集とIDATは同日に行われた。血清は25のアレルギーに特異的なIgEの検出のために、独自に開発した抗CCD IgE阻害剤による治療の前後で測定された。各犬のデータは、獣医師が記入したアンケートによって収集された。

結果 – 検査方法間の相関は、抗CCD IgE阻害剤投与前(Spearman's ρ , $q = 0.2092$)と投与後 ($q = 0.3042$)で良好であった。シクロスポリンの投与量 ($P = 0.003$) は使用期間とは無関係に、また、ロキベトマブの使用期間 ($P = 0.001$) は投与量とは無関係に、すべてのアレルギータイプにおいてIgE濃度の統計的に有意な低下と関連していた。

結論と臨床的妥当性 – これまでの報告とは異なり、本研究では、抗CCD IgEを阻害した後もSATとIDATの相関性は変わらないことが示された。シクロスポリンの投与量およびlokivetmabの治療期間は、SAT中のIgE濃度に未解明の影響を及ぼす可能性がある。

摘要

背景 – 血清学過敏原検測(SAT)广泛用于制定特异性犬的過敏原特异性免疫治療。交叉反应性碳水化合物決定簇(CCD)特异性血清免疫球蛋白(Ig)E可产生假阳性反应，导致SAT和皮内過敏原試驗(IDAT)結果之间的差异。

目的 – 主要目的是确定在商业检测中抑制抗CCD IgE是否改善与IDAT的相关性。次要目的是评估犬和临床特异性因素、环境因素、假定過敏原暴露和既往用藥对皮内和SAT反应性的影响。

动物 – 211只客户拥有的犬来自北美的8个皮膚专科診所。

方法和材料 – 在同一天进行血清样本采集和IDAT。在用抗CCD IgE的专用抑制剂处理前后，測定血清中25种過敏原特异性IgE的检测。通过兽医人员填写的调查问卷收集每只犬的数据。

結果 – 抗CCD IgE抑制前(斯皮爾曼相關係數, $\rho = 0.2092$)和抑制后($\rho = 0.3042$)，检测方式之间的相关性一般。环孢素剂量($P = 0.003$)与使用持续时间和洛基维特单抗使用持续时间($P = 0.001$)无关，与给药剂量无关，与所有過敏原类型中IgE浓度的统计学显著性降低相关。

結論和臨床相关性 – 与之前的报告相反，本研究表明抗CCD IgE抑制后SAT和IDAT之间的相关性不变。在SAT期间，环孢素剂量和洛基维特单抗治療持续时间可能对IgE浓度的影响尚需探索。

Resumo

Contexto – O teste sorológico de alérgenos (TAS) é amplamente utilizado para formular imunoterapia alérgeno-específica para cães atópicos. A imunoglobulina (Ig)E sérica específica para determinantes de carboidratos de reação cruzada (CCD) pode produzir reações falso-positivas, criando discrepância entre os resultados do TAS e do teste de alérgeno intradérmico (TAID).

Objetivos – O objetivo principal era determinar se a inibição de IgE anti-CCD em um ensaio comercial melhorava a correlação com o TAID. O objetivo secundário foi avaliar a influência de fatores específicos do cão e da clínica, fatores ambientais, suposta exposição a alérgenos e medicamentos anteriores na reatividade intradérmica e TAS.

Animais – Duzentos e onze cães de propriedade de clientes foram inscritos em oito clínicas de especializadas dermatologia norte-americanas.

Métodos e materiais – A coleta das amostras de soro e o TDAI foram realizadas no mesmo dia. Os soros foram testados para detecção de IgE específica para 25 alérgenos, antes e depois do tratamento com um inibidor patenteado de IgE anti-CCD. Os dados de cada cão foram coletados por meio de um questionário preenchido pela equipe veterinária.

Resultados – A correlação entre as modalidades de teste foi razoável antes (ρ de Spearman, $\rho = 0,2092$) e após ($\rho = 0,3042$) a inibição de IgE anti-CCD. A dose de ciclosporina ($P = 0,003$), independente da duração do uso e a duração do uso de lokivetmab ($P = 0,001$), independente da dose administrada, foram associadas a diminuições estatisticamente significativas nas concentrações de IgE em todos os tipos de alérgenos.

Conclusões e relevância clínica – Ao contrário de relatos anteriores, este estudo demonstrou correlação inalterada entre TAS e TAID após a inibição de IgE anti-CCD. A dose de ciclosporina e a duração do tratamento com lokivetmab podem ter efeitos inexplorados na concentração de IgE durante o TAS.

Table S1: Questionnaire filled out by veterinary personnel for each enrolled subject

Serological Study – Patient Data Form

Date of test: _____

***Patient's study number:** _____ **Patient's medical record number:** _____

*Study number must match the number written on the serum tube and Greer submission form

A. Eligibility – drug withdrawal

- a. Oral steroids used within the past 30 days: ☐ no ☐ yes
- b. Topical steroids used within the past 14 days. ☐ no ☐ yes
- c. Parenteral/injectable steroids used within the past 90 days. ☐ no ☐ yes
- d. Oral or topical antihistamines used within the past 14 days. ☐ no ☐ yes
- e. Oral essential fatty acids used within the past 14 days. ☐ no ☐ yes

*Note: if you answered “yes” to any of these drug withdrawal questions, the patient is NOT eligible for enrollment in this study.

B. Signalment data:

- 1. Age (or date of birth): _____ (must be at least 10 months to participate)
- 2. Duration of clinical signs (or date of onset): _____
- 3. Breed (or dominant mixed breed): _____
- 4. Sex (M, F, MC, FS): _____
- 5. Body weight (kg): _____ (must be at least 8kg to participate)

C. Historical data:

- 1. Housing: ☐ Predominantly Indoor ☐ Predominantly Outdoor ☐ Equal mix
- 2. Clinical signs: (please check one of the following):
 - a. ☐ Seasonal. (list months affected): _____
 - b. ☐ Non-seasonal.
 - c. ☐ Perennial with seasonal exacerbations (list peak months): _____
- 3. Parasitism:
 - a. Fleas seen within the 3 months prior to today's test: ☐ no ☐ yes
 - b. Diagnosed with scabies within the 3 months prior to today's test: ☐ no ☐ yes
 - c. Intestinal parasites within last three months? ☐ no ☐ yes ☐ unknown
 - d. Anti-parasitic treatments received:
 - i. Current on heartworm preventative? ☐ no ☐ yes ☐ unknown
If yes, please list product name _____
 - ii. Current on flea/tick preventative? ☐ no ☐ yes ☐ unknown
If yes, please list product name _____
 - iii. Any additional anti-parasitic drugs used (eg., for intestinal parasites)?
If yes, please list and provide last date given: _____

4. *Malassezia*: Diagnosed with infection/overgrowth within the past 3 months?

a. Skin infection ☐no ☐yes ☐unknown

b. Ear canal infection ☐no ☐yes ☐unknown

5. Adverse food reaction:

a. AFR is suspected to be part of this dog's allergic complex: ☐no ☐yes

b. Has an elimination diet trial been completed? ☐no ☐yes ☐unknown

i. If yes, duration of trial (in weeks): _____

ii. Type/brand of food used: _____

6. Cat dander. Has this dog lived with a cat within the past 3 months?

☐no ☐yes ☐unknown

D. Current therapies:

Has this dog received any of the following drugs within the past 30 days?

(These DO NOT preclude enrollment)

a. Cyclosporine ☐no ☐yes

If yes, please list dose and duration of use: _____

b. Oclacitinib ☐no ☐yes

If yes, please list dose and duration of use: _____

c. Lokivetmab ☐no ☐yes

If yes, please list dose and duration of use: _____

d. Has this dog received allergen-specific immunotherapy within the past 6 months?

☐no ☐yes (if yes, how recently?) _____

Please submit both pages of this form, along with the skin test results sheet, to the primary investigator at: XXX-XXX-XXXX (fax) or e-mail to: XXXXX@XXXXX

It is NOT necessary to send the client consent form. Please DO retain the consent form in the pet's medical record for 3 years.