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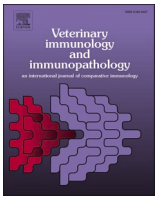

Evaluation of sensitization to the crude extract of *Dermatophagoides farinae* and its derived allergens, Der f 2 and Zen 1, in dogs with atopic dermatitis in Southern Brazil

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ABSTRACT

Background: Atopic dermatitis is associated with the production of IgE antibodies against environmental allergens and allergens of the house dust mite*Dermatophagoides farinae* are frequently implicated in the disease.
Objectives: We aimed to observe the allergen-specific IgE against crude*D. farinae*, Der f 2 and Zen 1 in dogs with atopic dermatitis and report if these dogs are in contact with material that could shelter mite allergens.
Methods: 100 dogs with clinical diagnosis of atopic dermatitis were included after exclusion of other forms of pruritic skin disease and dogs that already received specific or non-specific immunotherapy. These dogs were of different breeds and ages and they were presented at a veterinary teaching hospital and a private service of veterinary dermatology, both located in Curitiba, Southern Brazil. At the time of anamnesis, some questions were applied to know the possibility of these dogs having had contact with furniture and textile material which could shelter house dust mites. Sera samples were obtained and further analyzed by ELISA assay to measure serum IgE levels against these allergens with an established cut-off of 0.200 IgE optical density.
Results: The allergen-specific IgE positivity against crude*D. farinae* (92 %) and Zen 1 (77 %) was higher than Der f 2 (56 %). There was a correlation in sensitization to crude *D. farinae* and Zen 1 that was not observed between crude *D. farinae* and Der f 2 and Der f 2 and Zen 1. The sensitization to *D. farinae* and its allergens was associated with an unrestricted exposition to furniture and textile material.
Conclusion & clinical relevance: dogs with atopic dermatitis are frequently sensitized to *D. farinae* and its allergens, Der f 2 and Zen 1, may be considered major allergens in these dogs. Zen 1 may be the main allergen responsible for the sensitization to crude *D. farinae*.

1. Introduction

Atopic dermatitis is a genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical signs and is commonly associated with immunoglobulin E (IgE) antibodies to environmental allergens such as pollens, molds, and house dust mites (Hensel et al., 2015). Within house dust mites, the genus *Dermatophagoides* is overrepresented, mainly by the species *D. pteronyssinus* and *D. farinae* (Arlian and Platts-Mills, 2001). These species are found commonly in house dust, mainly in carpets, mattresses, and other textile material (Agratorres et al., 1999; Arlian and Morgan, 2003), where high humidity, moderate temperature, and food availability are ideal for their reproduction and development (Arlian and Morgan, 2003).

Sensitization to *D. farinae* allergens is common in dogs with atopic dermatitis (Nuttall et al., 2006). Previous studies observed that repeated epidermal applications of *D. farinae* extracts led to the development of clinical signs, mainly through the Th2 biased immune response, reflecting the importance of this response on mite allergen sensitization (Yamamoto et al., 2007; Pucheu-Haston et al., 2008). According to the World Health Organization Allergen Nomenclature Sub-Committee and the International Union of Immunological Societies, 31 *D. farinae*–derived allergens have been characterized and are listed in a database available at www.allergen.org. Among them, high molecular weight (HMW) allergens of groups 15 and 18 are classically considered

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major in atopic dermatitis in dogs (McCall et al., 2001; Nuttall et al., 2001; Weber et al., 2003). Der f 2, which has a sequence of 129 amino acids and a molecular weight of 14 kD (Trudinger et al., 1991), is considered a major allergen in dogs with atopic dermatitis in Japan (Yamashita et al., 2002), Spain (Moya et al., 2016), and England (Patel, 2019) but minor in Malaysia (Chan et al., 2019), European countries (France and Switzerland) and the United States (Olivry et al., 2017a,b). Group 2 allergens are classified as lipid-binding proteins with high IgE affinity and found in mite fecal pellets (Thomas, 2015). However, other authors stated that this group is found in low concentrations in feces and, therefore, are derived from sources other than the intestines (Colloff, 2009). Group 2 allergens show structural homology with myeloid differentiation factor 2 (MD-2) and have a high binding capacity with toll like receptor 4 and pathogen-associated molecular pattern, mainly bacterial lipopolysaccharides (Ichikawa et al., 2009). This feature can make group 2 allergens activate the innate immune response and lead to a Th2 response (Ichikawa et al., 2009; Scheurer et al., 2015).

In addition, Zen 1 is another HMW allergen from *D. farinae* (188 kD) that is major in atopic dogs in Japan (Tsukui et al., 2008), England (Patel, 2019), France, Switzerland, and the United States (Olivry et al., 2017a,b), but can be considered minor in atopic dogs from Malaysia (Chan et al., 2019).

The aim of this study was to evaluate the presence of allergen-specific IgE against the crude extract of *D. farinae*, Der f 2 and Zen 1, in dogs with atopic dermatitis.

2. Material and methods

This study was approved by the Ethics Committee on the Use of Animals of the Pontifical Catholic University of Paraná (PUCPR) (no. 01185).

2.1. Inclusion criteria

Dogs with a clinical diagnosis of atopic dermatitis and a history of chronic non-seasonal and primary pruritus were included according to established criteria after the exclusion of those with other pruritic dermatoses (Favrot et al., 2010). None of the included dogs had a history of previous immunotherapeutic treatment. Dogs of different breeds and ages were randomly selected from the Service of Dermatology and Allergy of Companion Animals of PUCPR and from a private clinic (Veterinary Dermatology Clinic – Dermatovet), city of Curitiba, Paraná State, Brazil.

2.2. Serum samples

A 5-mL blood sample was collected by cephalic or jugular venipuncture. The serum of each sample was obtained and a 0.5-mL portion was separated out, stored in a microtube, and kept frozen at -20 °C until analysis.

Environmental data

During anamnesis, environmental data were collected for each dog, including:

a) Did the dog have contact with carpets?
b) Was the dog dressed in clothes?
c) Did the dog climb beds and couches?
d) Did the dog have its own bed?
e) Did the dog have its own blanket?

2.3. ELISA

The ELISA assays were performed at the Central Research Laboratory - ZENOAQ, Fukushima, Japan, using horseradish peroxidase-labelled anti-dog IgE monoclonal antibody (Bethyl; A40−15 P), *D. farinae* crude extract (Greer Laboratories, USA), recombinant Der f 2, and native Zen 1 (both from Zenoaq). The methodology used here was described previously (Chan et al., 2019), and the cut-off value was set to OD = 0.2. The basis for setting this cut-off value is that the value is equivalent to twice of the ELISA using healthy beagle dog`s sera that were performed before. The 0.2 OD in this ELISA in which the dog IgE (Bethyl) is directly immobilized on the plate correspond to 0.2 ng/mL. If the specific-igE in sera was estimated to be less than 0.2 ng/mL, it was considered an extremely small value.

2.4. Statistical analysis

The chi-squared test was used to compare the proportions of dogs positive for each specific IgE. The data are expressed as proportions, considering a 95 % confidence interval and 5% significance level ($p < 0.05$). The correlations between the IgE optical densities (OD) for Zen 1 and Der f 2 in relation to crude *D. farinae* and between Der f 2 and Zen 1 were calculated using Spearman's rank coefficients considering a 5% significance level ($p < 0.05$). The correlation coefficient was classified according to previously studies (Mukaka, 2012; Patel et al., 2019).

Allergen-specific IgE OD of positive dogs with access to different environmental material were compared using Kruskal-Wallis test. The optical densities were presented as means, with respective 95 % confidence intervals, considering a significance level of 5% ($p < 0.05$).

3. Results

3.1. Epidemiological data

Altogether, 100 dogs were included representing 23 different breeds, with a predominance of Lhasa Apso, Shih Tzu, Maltese, French bulldog, and mongrel. Subjects' ages were 11 months to 14 years (median, 5 years). Regarding sex, 44 % were male and 56 % were female.

3.2. Serology

The seropositivity results for each allergen are shown in Fig. 1. Of the 100 dogs, 92 (92 %) were positive to crude *D. farinae*, 56 (56 %) to Der f 2, and 77 (77 %) to Zen 1. The OD value for each allergen is shown in Fig. 2. There was a statistically significant difference in positivity to crude *D. farinae* with Der f 2 and Zen 1 ($p < 0.001$ and $p = 0.0057$, respectively).

We observed a correlation between IgEs specific for crude *D. farinae* and Zen 1 (Spearman's $r = 0.88$, $p < 0.001$; Fig. 3) but not between crude *D. farinae* and Der f 2 (Spearman's $r = 0.30$, $p < 0.002$; Fig. 4). Still, no correlation was noted between the OD values of IgEs for Zen 1 and Der f 2 (Spearman's $r = 0.22$, $p < 0.02$; Fig. 5).

3.3. Allergens and contact with environmental material

The results on the relationship between the IgE positivity to an allergen and the environmental exposure of the dogs are shown in Table 1. Only the regular use of clothes was applicable to <50 % of the dogs. Although there was no statistically significant difference between allergens, most of the Der f 2–positive dogs had contact with their owner's couches and beds (93 %), while most of the Zen 1–positive dogs had individual dog blankets (88 %).

4. Discussion

This study demonstrated that dogs with atopic dermatitis in Southern Brazil, are commonly sensitized to *D. farinae* as well as to its individual allergens, Der f 2 and Zen 1. A high sensitization to the crude *D. farinae* was also observed in previous studies in different locations (Day et al., 1996; Lian and Halliwell, 1998; Goicoa et al., 2008; Farmaki et al., 2012; Lauber et al., 2012; Bjelland et al., 2014; Olivry et al., 2017a,b; Patel,

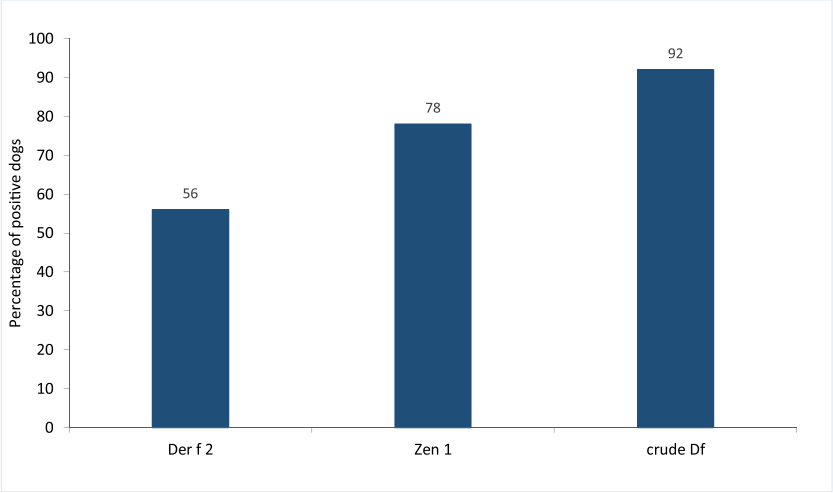

Fig. 1. Percentage of immunoglobulin E–seropositive dogs for crude *Dermatophagoides farinae*, Der f 2, and Zen 1.

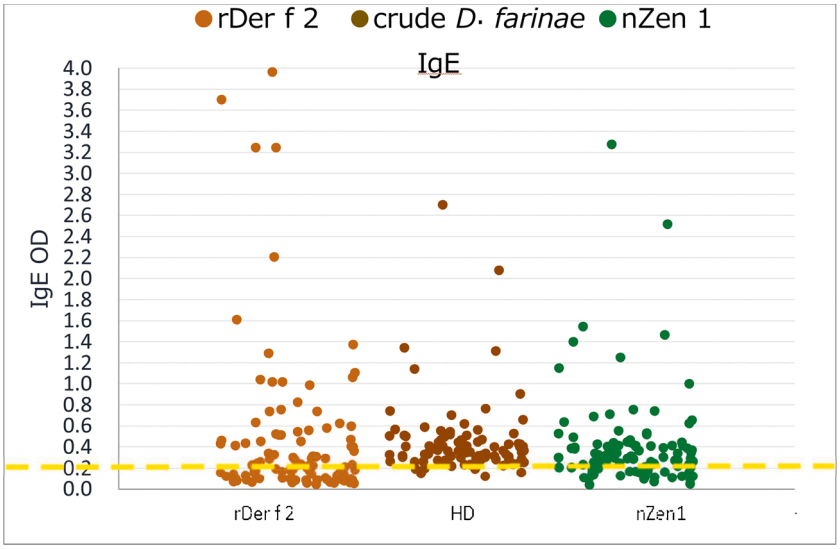

Fig. 2. IgE OD values for each allergen of a total of 100 serum samples from dogs with atopic dermatitis. HD: crude *Dermatophagoides farinae.*

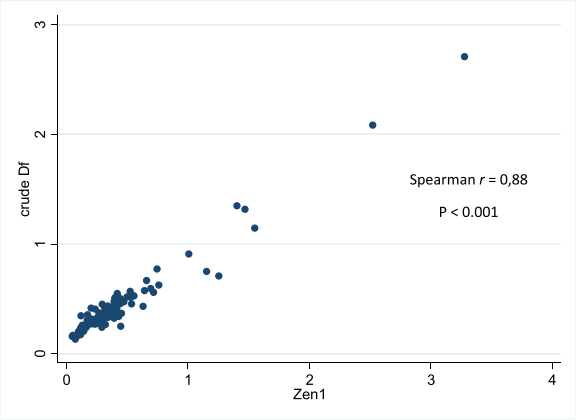

Fig. 3. Correlation between IgE OD values of crude *Dermatophagoides farinae* and Zen 1.

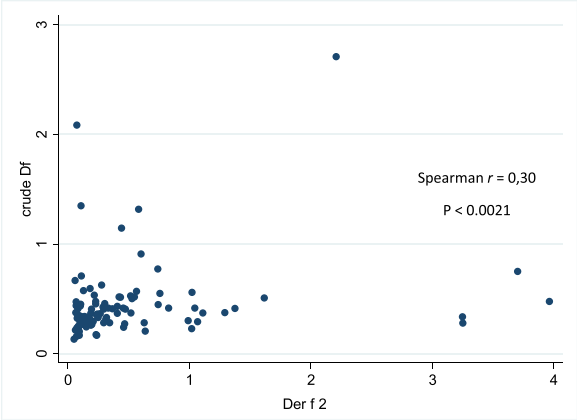

Fig. 4. Correlation between IgE OD values of crude *Dermatophagoides farinae* and Der f 2.

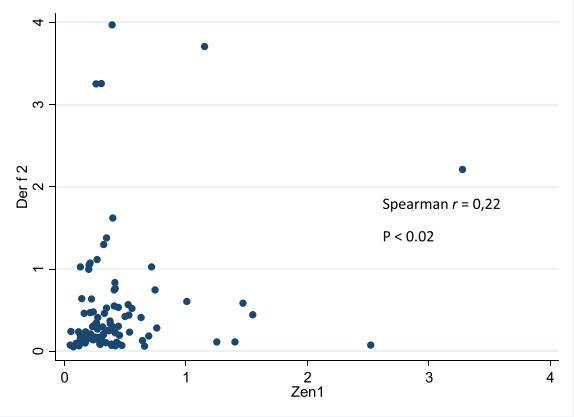

Fig. 5. Correlation between IgE OD values of Der f 2 and Zen 1.

Table 1
Percentage of dogs seropositive to each allergen and respective environmental exposure. Proportion of Der f 2–reactive dogs in contact with couches and beds versus the proportion of Zen 1 and crude *Dermatophagoides farinae*–reactive dogs in contact with the same objects (Kruskal-Wallis test, $p < 0.01$).

Furniture or textile material	Der f 2 (n = 56)	Zen 1 (n = 77)	crude *D. farinae* (n = 92)
Couch and bed	52 (93 %)*	65 (84 %)	76 (82 %)
Carpet	34 (61 %)	47 (61 %)	56 (61 %)
Beds for dogs	51 (91 %)*	65 (84 %)	78 (85 %)
Blankets for dogs	48 (86 %)	68 (88 %)	80 (87 %)
Clothes for dogs	25 (45 %)	32 (41 %)	35 (38 %)

* Proportion of Der f 2 positive dogs, in contact with couches, beds and beds for dogs, significantly different when compared to Zen 1 and crude Df positive dogs in contact with the same materials (Kruskal-Wallis test, p < 001).

2019), although different specific IgE assays have been used. In Brazil, previous studies showed that this mite species also plays an important role in atopic dog sensitization (Cunha et al., 2012; Pereira et al., 2015). In this study, we used a monoclonal anti-canine IgE antibody with high specificity, and it is unlikely that this high sensitization was due to immunoglobulin G (IgG) antibodies (Dérer et al., 1998). However, due to the fact that *D. farinae* and *D. pteronyssinus* mites can cross-react (Masuda et al., 1999), a portion of this high D. *farinae* sensitization could be due to *D. pteronyssinus* exposure.

In Brazil, *D. pteronyssinus* is commonly found in coastal zones with high humidity, whereas *D. farinae* is found in higher concentrations in dry and warm zones (Souza and Rosario, 2012). *D. pteronyssinus* allergens may be more common in the studied area (Dutra et al., 2001; Farias et al., 2015; Assunção et al., 2017), and it is important to highlight that group 2 allergens from *D. farinae* and *D. pteronyssinus* share up to 85 % of their amino acid sequences and that cross-reactions between them may occur (Masuda et al., 1999; Smith et al., 2001). However, dogs with atopic dermatitis may be more sensitized to *D. farinae* than other mites (Bensignor and Carlotti, 2002; Foster et al., 2003; Farmaki et al., 2012), even when they are most commonly found in the said environment (Farmaki et al., 2012)

This article shows that seropositivity to low molecular weight allergen Der f 2 can be considerably higher in the targeted area, this being the first study to demonstrate the importance of this allergen in the American continent. Allergens larger than *D. farinae* are usually associated with atopic eczema in dogs, particularly Der f 15 (98/109 kDa) and Der f 18 (60 kDa) (McCall et al., 2001; Nuttall et al., 2001; Weber et al., 2003). Low molecular weight allergens were considerably less important (Noli et al., 1996; Nuttall et al., 2001), except in Japan (Masuda et al., 1999; Yamashita et al., 2002).

A Japanese study was the first to demonstrate the importance of this Der f 2 allergen in dogs with atopic dermatitis in which 43 % of the 16 dogs showed lgE antibodies against Der f 2 in 1999 (Masuda et al., 1999) and 75 % of the 90 dogs were positive for Der f 2 in 2002 (Yamashita et al., 2002). In a previous Brazilian study, only 20 % of the 10 canine serum samples recognized bands between 12 and 17 kD, probably Der f 2 (Cunha et al., 2012). However, in 2016, a study showed a high prevalence of low molecular weight allergens of *D. farinae* in the serum of dogs with atopic dermatitis in Brazil using western blot assay with canine IgE monoclonal antibody (Possebom et al., 2016). The results of this study demonstrated 100 % of the serum-recognizing bands between 21 and 31 kD, possibly Der f 1.

Recently, with the standardization of highly specific techniques using purified and recombinant allergens in addition to monoclonal antibodies, these low molecular weight allergens are being highlighted, with 100 %, 97 % and 48.4 % of dogs showing IgE against Der f 2 in Spain, England and Malaysia, respectively (Moya et al., 2016; Patel, 2019; Chan et al., 2019). However, in the United States (North Carolina), France, and Switzerland, the Der f 2 allergen seems not to promote sensitization in dogs (Olivry et al., 2017a,b). This discrepancy between studies from different regions shows the importance of geographical variations promoting the genetics and allergenicity of these allergens.

A high seropositivity to the Zen 1 allergen, a HMW allergen of 188 kDa (range, 150–250 kDa), was also observed in this study. This allergen was first identified in a Japanese study in 2008 in which the sera of atopic dogs recognized a 188-kD band of *D. farinae* that did not show homology with other allergens (Tsukui et al., 2008). This allergen was characterized and sequenced and was provisionally named Zen 1, which is still the name used today. In the first published study measuring IgE against native Zen 1, of the 33 dogs with spontaneous atopic dermatitis in the United States, 88 % were seropositive, while 90 % of 29 dogs were seropositive in Europe (France and Switzerland), characterizing this new allergen as major in these regions (Olivry et al., 2017a,b). After that, Zen 1 was also considered major in Southeast England, where 76 % of 59 dogs tested positive (Patel, 2019). Although Zen 1 may also play a role in sensitization in Malaysian atopic dogs, only 29.8 % were IgE positive in a recent previous study (Chan et al., 2019). In addition, 175-kDa proteins were identified in a *D. farinae* extract in Spain that could be Zen 1 (Moya et al., 2016). Interestingly, a previous study in Brazil showed that serum from 5 of 10 dogs recognized 225-kD bands by western blotting that could also be Zen 1, but no further investigation was performed (Cunha et al., 2012). Dogs with atopic dermatitis may produce more IgG antibodies, mainly IgG1, against a 180-kDa protein of *D. farinae* (Hou et al., 2006).

Little is known about Zen 1 allergen, and its function is unclear. Its sequence differs from that of other *Dermatophagoides* allergens, but it may share conformational surface epitopes with Der f 15. Both proteins also converge in highly O-glycosylated portions, which could lead to cross-reactivity (Olivry et al., 2017a,b). This cross-reactivity may explain, in this study, the high positivity to Zen 1 compared to Der f 2 once Der f 15 was discovered to be a major allergen in Brazil (Cunha et al., 2012; Possebom et al., 2016). Also, *Toxocara canis* larvae are covered by heavily O-glycosylated mucins and it is possible that *T. canis* infection may lead to false-positive results to these house dust mite allergens (Fischer et al., 2018a,b).

In the present study, the IgE OD values between the crude *D. farinae* and Zen 1 were significantly correlated, but the same did not occur between the crude extract and Der f 2. Thus, it is likely that the seropositivity to the crude extract was related to HMW allergens such as Zen 1 as observed by other authors (Olivry et al., 2017a,b; Patel, 2019). There was also no correlation between Der f 2 and Zen 1, which may be due to the fact that Zen 1 arises from a different biological source than Der f 2, and it is likely that they do not cross-react.

The role of IgE antibodies in the physiopathology of atopic dermatitis still requires clarification. The physiological and pathophysiological effects of IgE are manifested through the interaction with its high

affinity (FcεRIα) and low affinity (CD23) receptors on the surface of effector cells, mainly mast cells, basophils, and eosinophils, promoting the release of cytokines and inflammatory mediators and regulating the production of antibodies by plasma cells and B lymphocytes and in the antigen presentation by B lymphocytes and dendritic cells (Hammerberg, 2013). It is known that non-atopic dogs may present with high levels of IgE to determined allergens (Lian and Halliwell, 1998; Roque et al., 2011; Lauber et al., 2012). However, some experimentally sensitized dogs may present low levels of allergen specific IgE (Okayama et al., 2011). This controversial correlation between IgE levels and clinical disease can be explained by the possibility of just the variable portion of this molecule being directed against environmental allergens (Pucheau-Haston et al., 2015). Therefore, the fact that these dogs show serological levels of IgE above the cut-off implies sensitization but not necessarily the development or onset of clinical signs since the immunopathogenesis of the disease is highly complex. IgE can be functionally heterogeneous, with fractions that differentiate in allergenicity, mobility, and ability to bind to other molecules (Pucheau-Haston et al., 2015). However, the good response seen in humans with atopic dermatitis after the use of a parenteral anti-IgE monoclonal antibody demonstrates the important role that this antibody has in the pathophysiology of the disease (Sheinkopf et al., 2008).

The importance of *D. farinae* allergens in canine atopic dermatitis has also been demonstrated by the good response to an allergen-specific immunotherapy with pullulan-conjugated recombinant Der f 2. After a few months of immunotherapy, these dogs showed clinical improvement, decreased lesion (CADESI) and pruritus scores, and decreased use of medications (Olivry et al., 2017a,b; Kawano and Mizuno, 2017; Fischer et al., 2018a,b). It is relevant that such Der f 2-responsive dogs including *D. farinae*–sensitized dogs and multiple allergen-sensitized dogs benefited from these therapeutic options (Kawano and Mizuno, 2017). Therefore, it is feasible that specific anti-Der f 2 immunotherapy stimulates immunoregulatory routes, minimizing the Th2 response. In addition, the adjuvant pullulan is a polysaccharide that reduces the allergenicity and allergen recognition by IgE in addition to promoting Th1 cytokines (Mehvar, 2003; Wang et al., 2016).

Although there are different routes of environmental allergen sensitization in atopic dogs, epidermal penetration is the main route, occurring through direct contact with an allergen (Marsella et al., 2006). Despite these mites also being found on dog hairs (Randall et al., 2005; Farias et al., 2015; Assunção et al., 2017), the environmental load is significantly higher (Assunção et al., 2017).

The high allergen seropositivity demonstrated herein overlaps with a high exposure to reservoirs of house dust mites. It is notable that these mites are highly concentrated in household environments, mainly in places in which there is the presence of furniture and textile material such as couches, carpets, mattresses, and beds (Arlian and Morgan, 2003; Assunção et al., 2017). Most allergen-positive dogs in this study had free access to all household facilities and, therefore, contact with this type of material.

Still, most dogs reported here had contact with dog beds, which contain high concentrations of house dust mites and its allergens (Eaton et al., 1985; Randall et al., 2003; Raffan et al., 2005; Assunção et al., 2017). This high allergen concentration on these objects can be explained because it maintains adequate temperature and humidity as well as the presence of food and other substrates that provide a food source for the mites. Therefore, regular laundering of dog beds may help reduce the load of environmental allergens (Raffan et al., 2005).

Some dogs of the study were frequently dressed in synthetic or fleece clothes, which are also important reservoirs for mites and allergens (Neal et al., 2002; Teplitsky et al., 2008). A study showed that *D. farinae* mites are isolated in about 58 % of the clothes of humans with atopic dermatitis (Teplitsky et al., 2008). Still, the difference in the fabric may also affect mite concentrations. The fleece fabric seems to contain higher concentrations of mites than cotton fabric (Clarke et al., 2015). Therefore, it is feasible that this habit of wearing clothes may enhance the exposure to environmental allergens and encourage sensitization of susceptible dogs.

In conclusion, dogs with atopic dermatitis are commonly sensitized to *D. farinae* and its allergens Der f 2 and Zen 1. The sensitization to Zen 1 may be the main factor responsible for *D. farinae* sensitization. Frequent exposure to furniture and textile materials may favor this sensitization.

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Declaration of Competing Interest

Dr. Toshihiro Tsukui and Ms. Miyuki Kageyama are employees of Zenoaq.

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