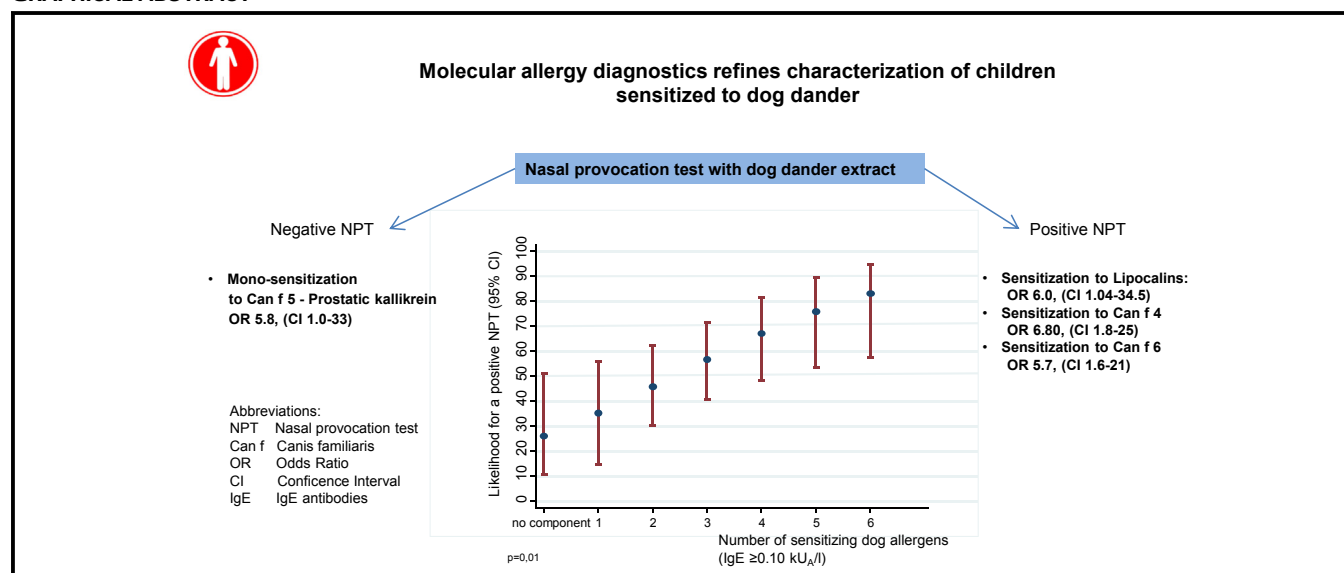


Molecular allergy diagnostics refine characterization of children sensitized to dog dander



Ulrika Käck, MD,^a Anna Asarnej, MD, PhD,^{b,c} Hans Grönlund, PhD,^d Magnus P. Borres, MD, PhD,^{e,f} Marianne van Hage, MD, PhD,^b Gunnar Lilja, MD, PhD,^a and Jon R. Konradsen, MD, PhD^{b,c} *Stockholm and Uppsala, Sweden*

GRAPHICAL ABSTRACT



Background: Sensitization to dog dander is an important risk factor for rhinoconjunctivitis and asthma but is not sufficient for diagnosing dog allergy. Molecular allergy diagnostics offer new opportunities for refined characterization.

Objectives: We sought to study the association between sensitization to all presently known dog allergen components and clinical symptoms of dog allergy in children evaluated by using nasal provocation tests (NPTs).

Methods: Sixty children (age, 10-18 years) sensitized to dog dander extract underwent NPTs with dog dander extract.

Measurement of IgE levels to dog dander and to Can f 1, Can f 2, Can f 3, and Can f 5 was performed with ImmunoCAP, and

measurement of IgE levels to Can f 4 and Can f 6 was performed with streptavidin ImmunoCAP. An IgE level of 0.1 kU_A/L or greater was considered positive.

Results: There was an association between sensitization to an increasing number of dog allergen components and a positive nasal challenge result ($P = .01$). Sensitization to lipocalins (odds ratio [OR], 6.0; 95% CI, 1.04-34.5), in particular Can f 4 (OR, 6.80; 95% CI 1.84-25.2) and Can f 6 (OR, 5.69; 95% CI, 1.59-20.8), was associated with a positive NPT result. Monosensitization to Can f 5 was related to a negative NPT result (OR, 5.78; 95% CI, 1.01-33.0).

From ^athe Department of Clinical Science and Education, Södersjukhuset, ^cthe Department of Women's and Children's Health, and ^dthe Department of Clinical Neuroscience, Karolinska Institutet, Stockholm; ^bthe Department of Medicine Solna Immunology and Allergy Unit, Karolinska Institutet and Karolinska University Hospital, Stockholm; ^eThermo Fisher Scientific, Uppsala; and ^fthe Department of Women's & Children's Health, Uppsala University.

Supported by the Swedish Asthma and Allergy Association's Research Foundation, the Stockholm County Council (ALF project and clinical post-doc), the Swedish Research Council, the Swedish Heart-Lung Foundation, the Swedish Cancer and Allergy Foundation, the Hesselman foundation, the Konsul Th C Bergh foundation, and the Karolinska Institutet.

Disclosure of potential conflict of interest: M. P. Borres is an employee of Thermo Fisher Scientific (Uppsala, Sweden). M. van Hage has received lecture fees from Thermo Fisher Scientific (Uppsala, Sweden) and consultancy fees from Biomay AG

(Vienna, Austria) and Hycor Biomedical. J. R. Konradsen has received material from Thermo Fisher to perform the IgE analysis in this project. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication October 6, 2017; revised April 23, 2018; accepted for publication May 18, 2018.

Available online May 29, 2018.

Corresponding author: Ulrika Käck, MD, Department of Clinical Science and Education, Södersjukhuset, Karolinska Institutet, S1, Sjukhusbacken 10, 118 83 Stockholm, Sweden. E-mail: ulrika.kack@sl.se.

The CrossMark symbol notifies online readers when updates have been made to the article such as errata or minor corrections

0091-6749/\$36.00

© 2018 American Academy of Allergy, Asthma & Immunology

<https://doi.org/10.1016/j.jaci.2018.05.012>

Conclusion: Sensitization to an increasing number of dog allergen components and to lipocalins is associated with dog allergy. Monosensitization to Can f 5 should not be regarded primarily as a marker for dog allergy. (J Allergy Clin Immunol 2018;142:1113-20.)

Key words: Allergy, Can f 1, Can f 2, Can f 3, Can f 4, Can f 5, Can f 6, children, dog, IgE, molecular allergology, nasal provocation test, sensitization

Dog allergy is one of the most common perennial airborne allergies among children.¹ Exposure to dogs causes not only rhinitis, asthma, or both but can also affect quality of life in the child with dog allergy.² Thus correct diagnosis and advice from the physician is essential.

Measurement of IgE antibodies to dog dander extract is an established diagnostic tool but has several limitations. Crude dog dander extracts have been shown to vary in allergen content, which can affect the accuracy of the test results.³ Moreover, a positive test result might be due to cross-reactivity with allergens from other furry animals and hence might be of uncertain clinical relevance.⁴

The introduction of molecularly based allergy diagnostics offers new opportunities for improved characterization and management.⁵ At the time of our investigation, there were 6 known dog allergens,⁶ and sensitization seems to start early in life.⁷ Four of these, Can f 1, Can f 2, Can f 4, and Can f 6, belong to the lipocalin protein family, which is present in dog dander, saliva, and urine.⁸⁻¹⁰ Can f 1 is considered a major allergen and is detected in 50% to 90% of patients sensitized to dog.⁵ Sensitization to Can f 2 and the more recently characterized Can f 4 and Can f 6 is less common. Can f 6 is highly cross-reactive with cat and horse lipocalins, whereas Can f 2 shows patient-dependent cross-reactivity with the cat lipocalin Fel d 4.¹¹⁻¹³ Can f 3, dog serum albumin, is a highly cross-reactive minor allergen detected in 15% to 35% of dog-sensitized patients.^{4,14} IgE reactivity to Can f 5, the male dog's prostatic kallikrein, has been found in up to 70% of dog-sensitized subjects, among whom 38% were not sensitized to Can f 1, Can f 2, or Can f 3, suggesting that there might be subjects allergic specifically to male dogs.¹⁵

Can f 7, Canine NPC2 protein (dog epididymal secretory protein), was recognized as a dog allergen in 2016¹⁶ after our study was conducted and is therefore not included in our analysis.

However, there is a need to further explore the clinical relevance of sensitization to different dog allergen components. The aim of this study was to analyze the patterns of IgE reactivity to dog allergen components among dog-sensitized children and, for the first time, to investigate the association between sensitization and clinical symptoms of dog allergy evaluated by using nasal provocation tests (NPTs) with dog dander extract.

METHODS

Patient population

A total of 105 patients were recruited from pediatric outpatient clinics in the Stockholm area. Inclusion criteria were age of 10 to 18 years and confirmed sensitization to dog dander based on a positive skin prick test response (wheal size > 3 mm), serum IgE antibodies to dog dander of greater than 0.10 kU_A/L, or both. The patients were included regardless of history of clinical symptoms to dog. Exclusion criteria were as follows: known impaired lung function of other cause than asthma and ongoing or completed

Abbreviations used

NPT: Nasal provocation test

OR: Odds ratio

ROC: Receiver operating characteristic

allergen-specific immunotherapy to furry animals. Six patients did not meet the inclusion criteria, and 39 declined participation after receiving detailed information. A total of 60 patients were included.

Written informed consent was obtained from all participants and their parents. The study was approved by the regional board of ethics at Karolinska Institutet (Dnr 2014/1453-31/4).

Interviews and standardized questionnaires

All children and their parents were interviewed according to a modified version of the standardized questionnaire used in the Environmental and Childhood Asthma study.¹⁷ The questionnaire included questions regarding demographic data, such as family history of allergy and asthma, exposure to dogs and other furry animals, history of asthma and rhinitis and other allergic manifestations, triggering symptoms, and medication and health care use.

NPTs

NPTs were performed with a commercially available dog dander extract, Aquagen 100,000 SQ-E/mL (ALK-Abelló, Copenhagen, Denmark), according to a modified Lebel protocol.¹⁸ The dog dander extract used was analyzed concerning concentrations of specific allergens by using competitive inhibition ELISA and was found to contain all 6 investigated components (See Table E1 in this article's Online Repository at www.jacionline.org).

One spray dose, 0.1 mL of dog dander extract (10,000 SQ-E), was deposited in each nostril. Symptoms during NPTs were scored according to the Lebel scoring scale before and 5, 15, and 30 minutes after administration by a trained research nurse.¹⁸ The scoring system identifies 3 cardinal symptoms: sneezing, rhinorrhea, and nose blockage, each graded on a scale from 0 to 3 points. In addition, nasal pruritus (1 point), ear pruritus (1 point), and eye symptoms (1 point) were registered. Maximum score was 12. According to Lebel et al,¹⁸ a score of 5 or greater was considered positive, and a score of 2 or less was considered negative.

NPTs were performed in a 2-step manner. Patients scoring 4 or less and who were clinically unaffected/modestly affected 30 minutes after spray dose 1 proceeded to spray dose 2 (100,000 SQ-E) in each nostril, and the scoring procedure was repeated. Patients with a maximum score of 2 to 4 after the second step of the NPT procedure were considered to have indecisive results. Treatment with oral antihistamines was withheld for at least 72 hours, and nasal steroids were withheld for 2 weeks before the NPT. No patient showed symptoms of allergy or infectious rhinitis before the investigation.

Serologic analysis

Samples of venous blood were collected, and IgE antibodies against dog dander extract (e5), Can f 1, Can f 2, Can f 3, and Can f 5 were analyzed by using ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden). Can f 4 and Can f 6 were produced as recombinant proteins according to previously published data.^{9,11} IgE levels to Can f 4 and Can f 6 were measured by coupling 5 µg of biotinylated antigen to streptavidin ImmunoCAP, as described by the manufacturer (Phadia AB/Thermo Fisher Scientific). All IgE determinations were analyzed by using the ImmunoCAP System (Phadia AB/Thermo Fisher Scientific), according to the manufacturer's instructions. Results are presented as kilounits of allergen per liter, where the cutoff for the presence of allergen-specific IgE was 0.10 kU_A/L or greater, which is the cutoff level in clinical settings.

TABLE I. Clinical assessment of children with negative, indecisive, and positive nasal challenges in relation to exposure, asthma, rhinitis, and other atopic manifestations

Parameter	All patients	Negative NPT result	Indecisive NPT result	P value, indecisive vs negative result	Positive NPT result	P value, positive vs negative result
No. of patients	60	21	14		25	
Mean age (y [SD])	13.1 (2.3)	13.0 (2.5)	12.5 (2.3)	.42	13.2 (2.1)	.67
Female sex, no. (%)	21 (35)	6 (29)	6 (43)	.38	9 (36)	.75
At least 1 parent with dog allergy, no. (%)	19 (32)	4 (19)	5 (36)	.27	10 (40)	.20
Exposure, no. (%)						
Dog at home	15 (25)	8 (38)	6 (43)	.78	1 (4)	.007
Cat at home	3 (5)	2 (10)	0 (0)	.51	1 (4)	.59
Exposure to horse	12 (20)	6 (29)	2 (14)	.43	4 (16)	.48
Patient smoking	2 (3)	2 (10)	0 (0)	.51	0 (0)	.20
Parental smoking	10 (17)	4 (19)	4 (29)	.69	2 (8)	.38
Smoking at home during infancy	14 (23)	8 (38)	3 (21)	.30	3 (12)	.20
Asthma, no. (%)						
Asthma diagnosis	51 (85)	15 (71)	12 (86)	.43	24 (96)	.036
Reported asthma at dog exposure	30 (50)	6 (29)	7 (50)	.20	17 (68)	.008
Reported asthma at cat exposure	18 (30)	4 (19)	4 (29)	.69	10 (40)	.19
Rhinitis, no. (%)						
Rhinitis	58 (97)	19 (90)	14 (100)	.23	25 (100)	.20
Reported rhinitis at dog exposure	41 (68)	8 (38)	9 (64)	.21	24 (96)	<.001
Reported rhinitis at cat exposure	37 (62)	12 (57)	6 (43)	.25	19 (76)	.17
Reported rhinitis, birch pollen	35 (58)	10 (48)	8 (57)	.80	17 (68)	.16
Reported rhinitis, grass pollen	28 (47)	8 (38)	6 (43)	.97	14 (56)	.23
Other atopic manifestations, no. (%)						
Food allergy, current or previous	39 (65)	10 (48)	11 (79)	.04	18 (72)	.09
Eczema, current or previous	38 (63)	11 (52)	12 (86)	.04	15 (60)	.60

Boldface indicates $P < .05$.

Statistics

All statistical analyses were performed with Stata statistical software (release 14.2; StataCorp, College Station, Tex).

Categorical data were compared by using the χ^2 test or Fisher exact test, where appropriate. The t test on log-transformed values was used for group comparisons of IgE levels (continuous variables). Odds ratios (ORs) with 95% CIs for associations were calculated by using logistic regression analysis. Adjustments for the 3 dog allergen protein types (lipocalin, serum albumin, and kallikrein) were performed to determine possible independent markers for a positive NPT result among the analyzed components. ORs for a positive NPT result in relation to the number of sensitizing dog allergens were estimated by using logistic regression models and 95% CIs. Fitted predicted probability estimates were plotted according to the number of IgE-reactive (≥ 0.1 kU/L) dog allergen molecules by using results from logistic regression. The diagnostic performance of IgE measurements to different components and dog dander extract was compared by using receiver operating characteristic (ROC) curve analysis.¹⁹

RESULTS

Baseline demographics

The mean age of the patients was 13.1 years, and boys were in the majority (65%). Thirty-two percent had at least 1 parent with reported dog allergy, and 25% were living in a home with a dog. The level of reported allergic symptoms was high; 97% reported any allergic rhinitis or conjunctivitis, and 68% reported rhinitis caused by dog. Eighty-five percent had a physician's diagnosis of asthma, and 50% reported asthma symptoms caused by dog exposure.

A large proportion also reported allergic symptoms when exposed to other furry animals. A total of 62% reported rhinitis, and 30% reported asthma caused by cat (Table I).

IgE reactivity and relation to reported symptoms

In accordance with the inclusion criteria, all children were sensitized to dog dander extract (median level, 11.5 kU_A/L; range, 0.19-219 kU_A/L; interquartile range, 3.15-43.0 kU_A/L). Among the components, sensitization to Can f 1 was most common, followed by sensitization to Can f 5, Can f 4, Can f 6, Can f 2, and Can f 3 (Fig 1, A). There was a large variation in IgE levels against the different components. The highest levels were found against Can f 1, Can f 2, and Can f 5 (Fig 1, B).

Reported dog-related rhinitis was most common among patients sensitized to Can f 3 (94% vs 59% among nonsensitized subjects, $P = .012$) and Can f 6 (86% vs 53%, $P = .007$). Patients sensitized to Can f 3 and Can f 6 also reported the highest frequencies of dog-induced asthma (69% vs 43% [$P = .08$] and 64% vs 38% [$P = .038$], respectively; Fig 1, A, and see Table E2 in this article's Online Repository at www.jacionline.org).

A majority of the patients were sensitized to several components. There was also a considerable overlap in sensitization to components from the 3 different protein groups: lipocalins, serum albumin, and prostatic kallikrein (Fig 2, A). When adjusting for sensitization to allergens from the different protein groups, there was no significant association between any of the groups and reported dog-induced rhinitis or asthma. However, among patients sensitized to all 3 protein groups, an association with reported dog-induced rhinitis (OR, 8.36; 95% CI, 1.00-69.5) was found (see Tables E3-E6 in this article's Online Repository at www.jacionline.org). Furthermore, sensitization to an increasing number of dog allergen components significantly increased the risk for reported dog-induced asthma and rhinitis (see Figs E1 and E2 in this article's Online Repository at www.jacionline.org).

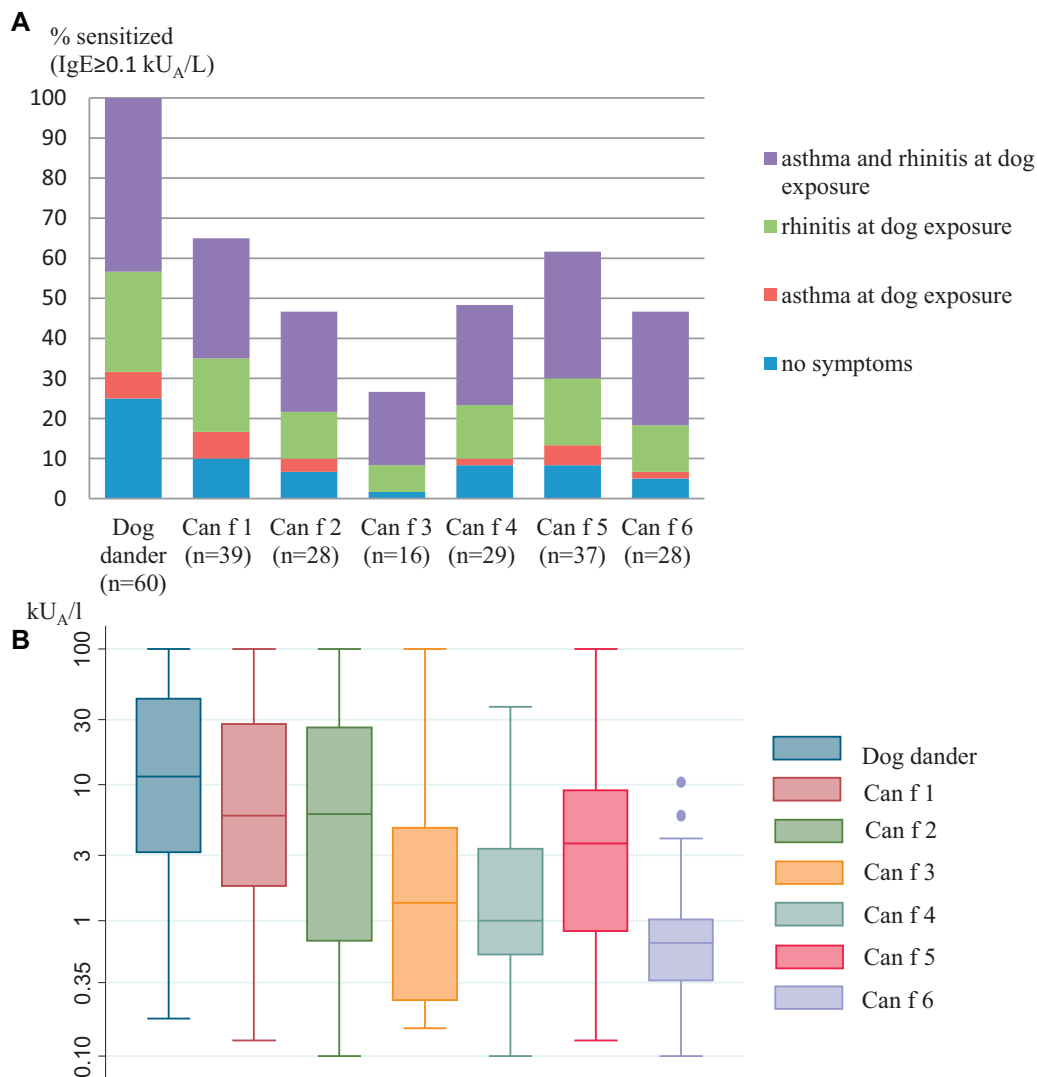


FIG 1. A, Frequencies of IgE reactivity to dog dander and components in relation to reported symptoms of rhinitis and asthma caused by exposure to dog. **B**, Levels of IgE (in kilounits of allergen per liter) to dog dander and components. Levels of greater than 100 kU/L were set at 100. Boxes indicate interquartile ranges, and whiskers indicate maximum/minimum levels among sensitized subjects.

NPTs and reported symptoms

Twenty-five patients were shown to have a positive NPT result with dog dander extract. Twenty-one patients scoring 2 or less were regarded as having negative results. Fourteen patients scored 3 to 4 but were still not clearly unaffected by the NPT, and therefore their results were considered indecisive.

Among the patients with a positive NPT result ($n = 25$) compared with a negative NPT result ($n = 21$), symptoms at dog exposure were more common: rhinitis (96% vs 38%, $P < .001$) and asthma (68% vs 29%, $P = .008$). Children with a positive provocation test result also reported greater frequencies of rhinitis and asthma when exposed to cats, although these were not statistically significant (Table I).

Only one of the 15 patients living with a dog had a positive NPT result, although there was no significant difference in IgE levels between those living with a dog and those not living with a dog (data not shown).

IgE reactivity and relation to positive NPT results

Sensitization to the lipocalins Can f 4 (OR, 6.8; 95% CI, 1.8-25) and Can f 6 (OR, 5.7; 95% CI, 1.6-21) and to the serum albumin Can f 3 (OR, 6.3; 95% CI, 1.2-33) was significantly associated with a positive NPT result in the unadjusted logistic regression analysis (Fig 3). In the unadjusted analysis lipocalins and serum albumin levels were associated with a positive NPT result, but after adjusting for the effect of the other protein groups, only sensitization to lipocalins was shown to be associated with an increased risk for a positive NPT result (Fig 3), whereas no such association was seen for serum albumin or prostatic kallikrein. Because multi-sensitization was common, we made a further analysis of the relation between sensitization profiles to the different protein groups and a positive NPT result (Fig 2, B). The highest OR for a positive NPT result was found among patients sensitized to all 3 protein groups. The lowest OR for a positive NPT result was found among subjects monosensitized to prostatic kallikrein, Can f 5. Patients

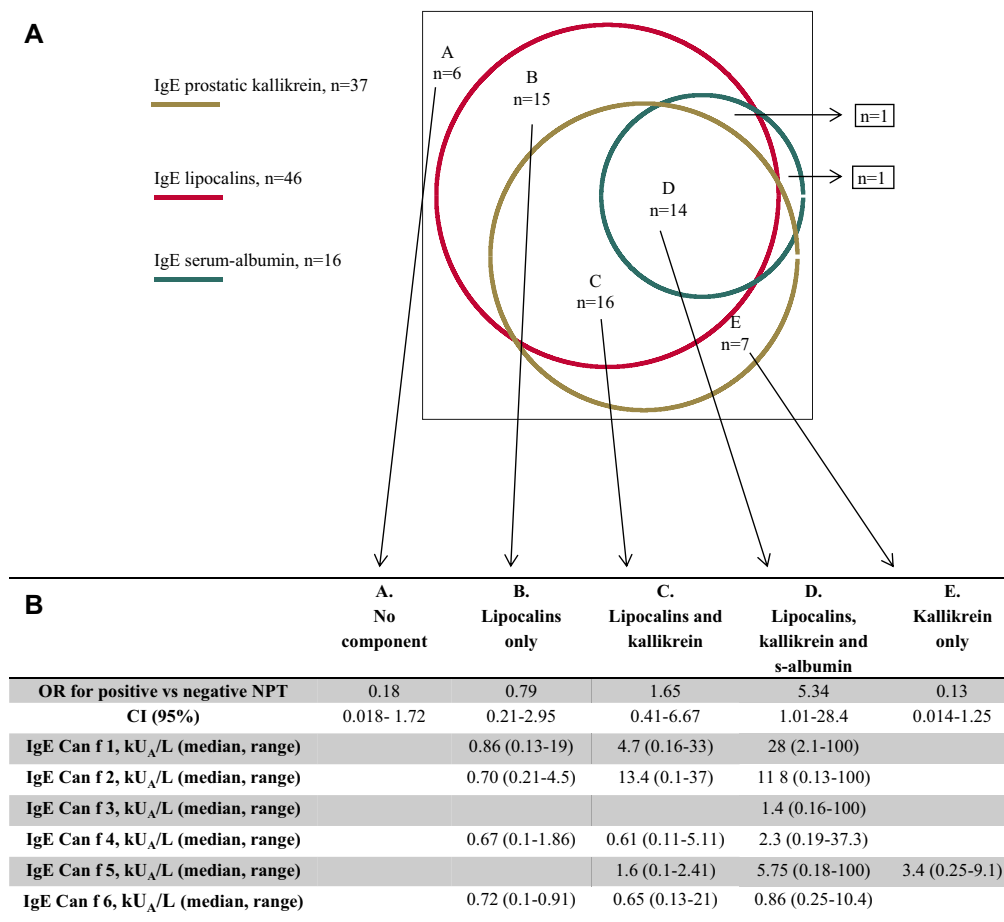


FIG 2. A, Venn diagram showing overlapping IgE reactivity to the 3 different protein groups: lipocalins, serum albumin, and prostatic kallikrein (n = 60). **B,** ORs for a positive versus negative NPT result are shown for the most common combinations of sensitization (n = 46). IgE levels are shown to the relevant components. Levels greater than 100 kU_A/L were set at 100 kU_A/L.

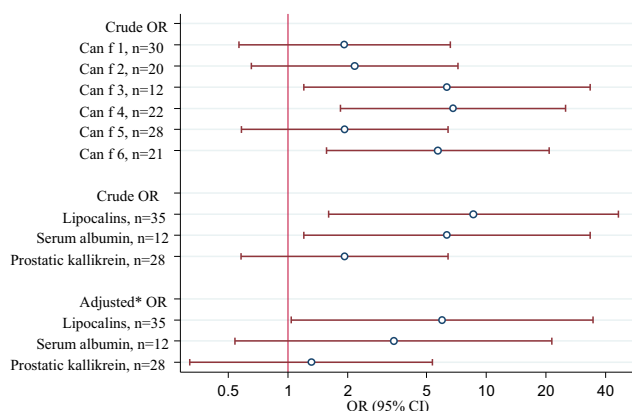


FIG 3. Crude ORs for a positive (n = 25) versus a negative (n = 21) NPT result in relation to IgE reactivity to dog allergen components (IgE ≥ 0.10 kU_A/L) and crude and adjusted ORs for IgE reactivity to the lipocalin, serum albumin, and prostatic kallikrein protein groups. *Adjusted for concomitant sensitization to allergens from other protein groups.

sensitized to dog dander only also showed a low OR for a positive NPT result (Fig 2, B).

Taking into account all 6 specific dog allergens, there was also a statistically significant association between an increasing number

of sensitizing dog components and the likelihood of a positive NPT result (Fig 4).

IgE sensitization and relation to a negative NPT result

When comparing patients with a negative NPT result (n = 21) with the rest of the study population (n = 39), we saw a significant association with a negative nasal challenge result in subjects monosensitized to Can f 5 (OR, 5.78; 95% CI, 1.01-33.0). Sensitization to dog dander only (no component) also showed a tendency toward a negative nasal challenge result (OR, 4.35; 95% CI, 0.73-26.1). No other sensitization profile could be linked to a negative NPT result.

Allergen-specific levels of IgE and relation to a positive NPT result

Median IgE levels against dog dander and all allergen molecules, except Can f 3, were greater among patients with a positive NPT result than among patients with no reaction (Table II). However, significance was only reached for IgE to Can f 1 and dog dander extract. To compare the diagnostic performance of IgE levels to different dog components, we

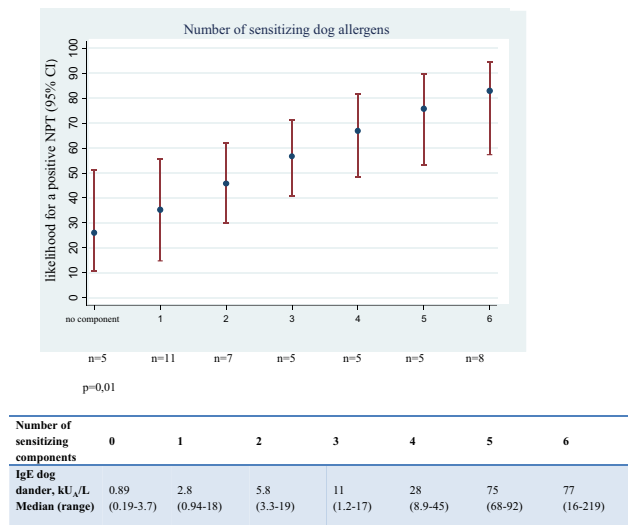


FIG 4. Likelihood (*y*-axis, percentage) of having a positive NPT result increases by 55% with every IgE-positive dog allergen component (*x*-axes). Analysis included 46 patients: 25 with a positive NPT result to dog dander extract and 21 with a negative NPT result to dog dander extract.

created ROC curves representing IgE to dog dander and to all 6 components in relation to a positive versus negative NPT result. There were no statistically significant differences between the different test performances (Fig 5).

DISCUSSION

In this study we investigated the usefulness of molecular allergology as a diagnostic tool in children with suspected allergy to dog. We analyzed IgE to Can f 1 to Can f 6 and explored associations between IgE levels and results from NPTs with dog dander extract, the latter regarded as a gold standard within allergy diagnostics.

The likelihood of a positive NPT result was associated with sensitization to an increasing number of components. When analyzing single components, we found that a positive NPT result was associated with the dog allergens Can f 3, Can f 4, and Can f 6, whereas no such association was found with IgE to Can f 1, Can f 2, or Can f 5. Furthermore, sensitization to lipocalins was associated with a positive NPT result, and this association was strengthened in children sensitized to allergens from all 3 protein groups: lipocalins, serum albumin, and prostatic kallikrein. Finally, we found an association between a negative nasal challenge result and monosensitization to Can f 5.

ROC curves show that IgE to single components is not superior to IgE to dog dander in diagnosing dog allergy; however, our results demonstrate that revealing patterns of sensitization, as described above, add valuable clinical information in the diagnostic work-up when currently available diagnostic methods are inconclusive.

The present study highlights the significance of lipocalins as important sensitizers in patients with dog allergy. The presence of IgE to the minor lipocalin components Can f 4 and Can f 6 (together with the serum albumin Can f 3) conferred the greatest risk for a positive nasal challenge result. However, mean IgE levels toward Can f 4 and Can f 6 were generally low. Can f 6 has also shown extensive cross-reactivity with cat and horse lipocalins, and because the majority of the patients were multisensitized, this finding probably reflects cross-sensitization.

We have shown previously that sensitization to lipocalins is associated with asthma severity,² and Schoos et al²⁰ have shown recently that being sensitized to the lipocalin protein group was independently associated with asthma, but the study included only 2 components from dog, Can f 1 and Can f 2. Several studies have confirmed IgE reactivity to Can f 4 and Can f 6,^{9,21} but to our knowledge, no previous study has investigated the relation between sensitization to both Can f 4 and Can f 6 and clinical disease. Our conclusion is that sensitization to Can f 4 and Can f 6 can serve as a marker for clinically relevant dog allergy.

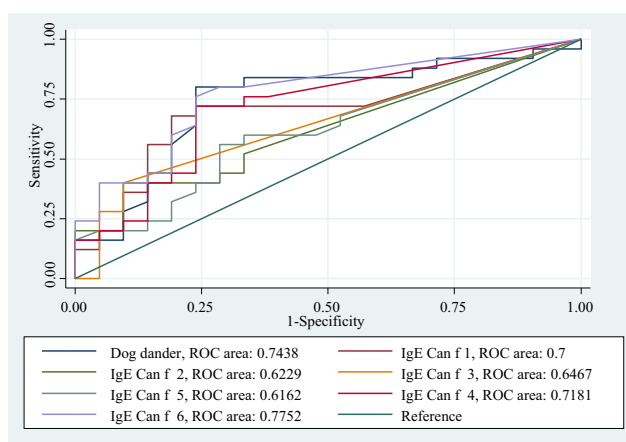
The presence of IgE to Can f 1 was not associated with a positive NPT result. This result appears to be in contrast with results from previous population-based studies, in which sensitization to Can f 1 early in life has been shown to be the best predictor for dog allergy²² and asthma later in childhood.⁷ In our selected cohort of dog dander-sensitized children, a majority of the patients were sensitized to Can f 1, which might explain why the increased risk of a positive NPT result was not significant for sensitization to this allergen. On the other hand, an increasing level of IgE to Can f 1 showed a strong relationship with a positive nasal challenge result, underlining the role of Can f 1 as a prominent dog allergen.

Sensitization to Can f 3 was associated with risk of a positive NPT result in the unadjusted analysis. However, when adjusting for concomitant sensitization to lipocalins and prostatic kallikrein, the association was not significant. Can f 3 was the least common sensitizing component in our study and mainly found among multisensitized subjects. The serum albumin Can f 3 is generally considered an allergen of less importance,²³ but results are contradictory. In a selected cohort of patients attending an allergy clinic, Uriarte and Sastre²⁴ found a strong association between sensitization to Can f 3 and severe respiratory symptoms. However, in a pediatric population-based study, sensitization to Can f 3 was uncommon and not associated with asthma.²⁵ Thus the dog serum albumin can serve as a marker for multisensitization rather than a marker for clinically relevant dog allergy.

Attention has been drawn to the prostatic kallikrein Can f 5 because a considerable proportion of dog dander-sensitized subjects are monosensitized to this allergen, and accordingly, these patients might be allergic to male dogs only.^{15,22} The first case report has been published recently, confirming that a patient with dog allergy monosensitized to Can f 5 was allergic only to male dogs. The study subject had a negative skin prick test response and no reaction to the ocular provocation test when using female dog dander extract, indicating tolerance to female dogs.²⁶ In our study population monosensitization to Can f 5 was associated with a negative nasal challenge result. This result is in line with the study by Asarnej et al,²² who found that monosensitization to Can f 5 was common among sensitized but asymptomatic children but in conflict with the study by Uriarte and Sastre,²⁴ who found a strong association between reported severe and persistent rhinitis and presence of IgE to Can f 5. In addition, Bjerg et al²⁵ found an association between IgE to Can f 5 and reported symptoms of rhinoconjunctivitis. However, in the studies by Uriarte and Sastre²⁴ and Bjerg et al,²⁵ adjustments for concomitant sensitization to all dog components was not performed, and provocation tests were not evaluated, which might in part explain the divergent results. Moreover, a strong independent relationship between the presence of IgE antibodies toward Can f 5 and asthma has been

TABLE II. Median level of IgE to different dog allergen components among all patients (n = 60)

Sensitizing Component	IgE level, median (IQR), all	IgE level, median (IQR), negative NPT result	IgE level, median (IQR), indecisive NPT result	IgE level, median (IQR), positive NPT result	P value, positive NPT result vs negative NPT result
Dog dander	11.5 (3.2-43)	4 (1.8-7)	14.5 (3-32)	19 (11-72)	.006
Can f 1	1.3 (0-13.5)	0 (0-1.8)	0.58 (0-13)	5.9 (0-31)	.002
Can f 2	0 (0-5.2)	0 (0-1.1)	0.18 (0-5.8)	0.13 (0-14)	.31
Can f 3	0 (0-0.16)	0 (0-0)	0 (0-0.16)	0 (0-0.5)	.37
Can f 4	0.1 (0-1.0)	0 (0-0)	0.1 (0-1)	0.4 (0-1.2)	.36
Can f 5	0.5 (0-5.6)	0.25 (0-3.7)	0.33 (0-2.1)	2.3 (0-5.8)	.32
Can f 6	0 (0-0.7)	0 (0-0)	0.11 (0-0.65)	0.4 (0-0.9)	.19

IQR, Interquartile range.**FIG 5.** ROC curves representing the true-positive rate (sensitivity) plotted in function of the false-positive rate (1 – specificity) for different IgE levels against Can f 1, Can f 2, Can f 3, Can f 4, Can f 5, Can f 6, and dog dander extract. Analysis included 46 patients: 25 with a positive NPT result to dog dander extract and 21 with a negative NPT result to dog dander extract.

reported, but also, this study has limitations regarding the number of allergens explored.²⁷ With this as a background, we suggest that Can f 5 could be regarded differently in the contexts of monosensitization and polysensitization.

Multisensitization to all 6 investigated components conferred the overall highest risk for a positive reaction. Among these multisensitized subjects, as well as among subjects sensitized to components from all 3 protein groups, IgE levels to dog dander and all lipocalins were generally greater, even though the ranges were broad. This association between multisensitization and dog allergy confirms and refines results from previous studies and can be a complement to diagnosing IgE to dog dander. Sensitization to more than 3 single lipocalins, prostatic kallikrein, and secretoglobulin components from furry animals has been associated with asthma severity.²⁸ Moreover, sensitization to several components from the same species has been shown to be a risk marker for clinical pet allergy.^{22,25} In this cohort multisensitization to dog components was common, and percentages of sensitization to minor allergens were generally greater than in previous studies. This is a possible reflection of the high prevalence of allergic manifestations among our patients. Thus our results confirm the effect of molecular spreading on clinical disease.

The major strength of this study is that nasal challenge has been performed and evaluated in all subjects. The NPT is a mainstay of allergy diagnostics because it directly proves the clinical

relevance of the tested allergen.²⁹ However, use of NPTs also has limitations, and international consensus guidelines do not exist.³⁰ Some authors argue that symptom scoring should be accompanied by an objective evaluation of nasal obstruction. In this study we used a recommended symptom score³¹ that has been shown to correlate with combined symptom scoring and rhinomanometry.³² Nonspecific nasal hyperreactivity might be a bias, but by exclusively comparing subjects with positive NPT results with those with clearly negative results, we minimize the risk of misclassifying patients with an unspecific reaction.

Using a semiquantitative scoring scale also has limitations. The symptom score was developed in adults, and the scoring might in part be biased by patients' expectations and, particularly in children, the ability to express symptoms.

Another limitation with nasal challenge is that the content of dog dander extracts can vary between producers and batches.³ To minimize the effect of this bias, we ascertained that all allergens were present in the extract used, and we used the same batch throughout the study. Another strength is that we have analyzed IgE antibodies using a quantitative measuring method (ImmunoCAP).

In conclusion, sensitization to lipocalins, as well as sensitization to an increasing number of dog allergen components, is associated with dog allergy. Also, high IgE levels to the lipocalin Can f 1 increases the risk of clinical symptoms at dog exposure. On the contrary, monosensitization to Can f 5 is not related to clinical symptoms and should not be regarded primarily as a marker for dog allergy. Accordingly, when clinical history and investigations are inconclusive, molecular allergology can add valuable clinical information in the diagnostic work-up of children with suspected dog allergy.

We thank all the children and families who participated. We also thank the staff at Barnforskningssentrum, Södersjukhuset, working with the MADOG project; research nurses Margareta Eriksson and Barbara Ekman; and biomedical scientist Carina Wallén.

Clinical implications: In children multisensitization to dog allergens, particularly to lipocalins, indicates clinically relevant dog allergy. Monosensitization to Can f 5 should not be regarded primarily as a marker for dog allergy.

REFERENCES

1. Ronmark E, Bjerg A, Perzanowski M, Platts-Mills T, Lundback B. Major increase in allergic sensitization in schoolchildren from 1996 to 2006 in northern Sweden. *J Allergy Clin Immunol* 2009;124:357-63, e1-15.

2. Konradsen JR, Nordlund B, Onell A, Borres MP, Gronlund H, Hedlin G. Severe childhood asthma and allergy to furry animals: refined assessment using molecular-based allergy diagnostics. *Pediatr Allergy Immunol* 2014;25:187-92.
3. Curin M, Reiningger R, Swoboda I, Focke M, Valenta R, Spitzauer S. Skin prick test extracts for dog allergy diagnosis show considerable variations regarding the content of major and minor dog allergens. *Int Arch Allergy Immunol* 2011;154:258-63.
4. Konradsen JR, Fujisawa T, van Hage M, Hedlin G, Hilger C, Kleine-Tebbe J, et al. Allergy to furry animals: new insights, diagnostic approaches, and challenges. *J Allergy Clin Immunol* 2015;135:616-25.
5. Borres MP, Ebisawa M, Eigenmann PA. Use of allergen components begins a new era in pediatric allergology. *Pediatr Allergy Immunol* 2011;22:454-61.
6. Nilsson OB, van Hage M, Gronlund H. Mammalian-derived respiratory allergens—implications for diagnosis and therapy of individuals allergic to furry animals. *Methods* 2014;66:86-95.
7. Nagao M, Borres MP, Sugimoto M, Petersson CJ, Nakayama S, Kuwabara Y, et al. Sensitization to secretoglobulin and lipocalins in a group of young children with risk of developing respiratory allergy. *Clin Mol Allergy* 2017;15:4.
8. de Groot H, Goei KG, van Swieten P, Aalberse RC. Affinity purification of a major and a minor allergen from dog extract: serologic activity of affinity-purified Can f I and of Can f I-depleted extract. *J Allergy Clin Immunol* 1991;87:1056-65.
9. Nilsson OB, Binnmyr J, Zoltowska A, Saarne T, van Hage M, Gronlund H. Characterization of the dog lipocalin allergen Can f 6: the role in cross-reactivity with cat and horse. *Allergy* 2012;67:751-7.
10. Polovic N, Waden K, Binnmyr J, Hamsten C, Gronneberg R, Palmberg C, et al. Dog saliva—an important source of dog allergens. *Allergy* 2013;68:585-92.
11. Madhurantakam C, Nilsson OB, Uchtenhagen H, Konradsen J, Saarne T, Høgbom E, et al. Crystal structure of the dog lipocalin allergen Can f 2: implications for cross-reactivity to the cat allergen Fel d 4. *J Mol Biol* 2010;401:68-83.
12. Mattsson L, Lundgren T, Olsson P, Sundberg M, Lidholm J. Molecular and immunological characterization of Can f 4: a dog dander allergen cross-reactive with a 23 kDa odorant-binding protein in cow dander. *Clin Exp Allergy* 2010;40:1276-87.
13. Hilger C, Swiontek K, Arumugam K, Lehnert C, Hentges F. Identification of a new major dog allergen highly cross-reactive with Fel d 4 in a population of cat- and dog-sensitized patients. *J Allergy Clin Immunol* 2012;129:1149-51.
14. Spitzauer S, Pandjaitan B, Soregi G, Muhl S, Ebner C, Kraft D, et al. IgE cross-reactivities against albumins in patients allergic to animals. *J Allergy Clin Immunol* 1995;96:951-9.
15. Mattsson L, Lundgren T, Everberg H, Larsson H, Lidholm J. Prostatic kallikrein: a new major dog allergen. *J Allergy Clin Immunol* 2009;123:362-8.
16. Khurana T, Newman-Lindsay S, Young PR, Slater JE. The NPC2 protein: a novel dog allergen. *Ann Allergy Asthma Immunol* 2016;116:440-6.e2.
17. Lodrup Carlsen KC, Haland G, Devulapalli CS, Munthe-Kaas M, Pettersen M, Granum B, et al. Asthma in every fifth child in Oslo, Norway: a 10-year follow up of a birth cohort study. *Allergy* 2006;61:454-60.
18. Lebel B, Bousquet J, Morel A, Chanal I, Godard P, Michel FB. Correlation between symptoms and the threshold for release of mediators in nasal secretions during nasal challenge with grass-pollen grains. *J Allergy Clin Immunol* 1988;82:869-77.
19. Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem* 1993;39:561-77.
20. Schoos AM, Kattan JD, Gimenez G, Sampson HA. Sensitization phenotypes based on protein groups and associations to allergic diseases in children. *J Allergy Clin Immunol* 2016;137:1277-80.
21. Rytönen-Nissinen M, Saarelainen S, Randell J, Hayrinen J, Kalkkinen N, Virtanen T. IgE reactivity of the dog lipocalin allergen Can f 4 and the development of a sandwich ELISA for its quantification. *Allergy Asthma Immunol Res* 2015;7:384-92.
22. Asaraoj A, Hamsten C, Waden K, Lupinek C, Andersson N, Kull I, et al. Sensitization to cat and dog allergen molecules in childhood and prediction of symptoms of cat and dog allergy in adolescence: a BAMSE/MeDALL study. *J Allergy Clin Immunol* 2016;137:813-21.e7.
23. Liccardi G, Asero R, D'Amato M, D'Amato G. Role of sensitization to mammalian serum albumin in allergic disease. *Curr Allergy Asthma Rep* 2011;11:421-6.
24. Uriarte SA, Sastre J. Clinical relevance of molecular diagnosis in pet allergy. *Allergy* 2016;71:1066-8.
25. Bjerg A, Winberg A, Berthold M, Mattsson L, Borres MP, Ronmark E. A population-based study of animal component sensitization, asthma, and rhinitis in schoolchildren. *Pediatr Allergy Immunol* 2015;26:557-63.
26. Schoos AM, Bonnelykke K, Chawes BL, Stokholm J, Bisgaard H, Kristensen B. Precision allergy: Separate allergies to male and female dogs. *J Allergy Clin Immunol Pract* 2017;5:1754-6.
27. Perzanowski MS, Ronmark E, James HR, Hedman L, Schuyler AJ, Bjerg A, et al. Relevance of specific IgE antibody titer to the prevalence, severity, and persistence of asthma among 19-year-olds in northern Sweden. *J Allergy Clin Immunol* 2016;138:1582-90.
28. Nordlund B, Konradsen JR, Kull I, Borres MP, Onell A, Hedlin G, et al. IgE antibodies to animal-derived lipocalin, kallikrein and secretoglobulin are markers of bronchial inflammation in severe childhood asthma. *Allergy* 2012;67:661-9.
29. Agache I, Bilo M, Braunstahl GJ, Delgado L, Demoly P, Eigenmann P, et al. In vivo diagnosis of allergic diseases—allergen provocation tests. *Allergy* 2015;70:355-65.
30. Pepper AN, Ledford DK. Nasal and ocular challenges. *J Allergy Clin Immunol* 2018;141:1570-7.
31. Dordal MT, Lluch-Bernal M, Sanchez MC, Rondon C, Navarro A, Montoro J, et al. Allergen-specific nasal provocation testing: review by the rhinoconjunctivitis committee of the Spanish Society of Allergy and Clinical Immunology. *J Investig Allergol Clin Immunol* 2011;21:1-12.
32. de Blay F, Doyen V, Lutz C, Godet J, Barnig C, Qi S, et al. A new, faster, and safe nasal provocation test method for diagnosing mite allergic rhinitis. *Ann Allergy Asthma Immunol* 2015;115:385-90.e1.

METHODS

ELISA

Concentrations of allergens in dander extracts were analyzed by using a competitive inhibition ELISA. Ninety-six-well MaxiSorp plates (Thermo Fisher Scientific) were coated overnight with 5 µg/mL goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, Pa) in PBS, pH 7.4. After rinsing 4 times with PBS containing 0.1% Tween 20 (Sigma-Aldrich), 200 µL per well of 1% BSA in PBS and 0.05% Tween 20 was added for 1 hour, followed by rinsing and incubation with allergen-specific mouse antibody for 2 hours. After rinsing, a mixture of a 50-µL sample and 50 µL of biotinylated antigen^{E1} was added, followed by 2 hours of incubation. The biotinylated antigen was titrated to provide an OD value in a sensitive range, typically between 0.8 and 1.0. After rinsing, the plates were incubated with streptavidin-horseradish peroxidase (Jackson ImmunoResearch) for 1 hour, and the substrate 3,3',5,5'-Tetramethylbenzidine Super Slow (Sigma-Aldrich), was added. The reaction was stopped with 0.1 mol/L sulfuric acid, and absorbance was measured at 450 nm and analyzed against an 8-step reference curve containing pure recombinant allergens, typically in the range of 2.5 to 320 ng/mL.

Antibody production

Assay antibodies were made as follows. Anti-Can f 1, Can f 2, and Can f 3 antibodies were produced by using conventional monoclonal methods,^{E2} with rCan f 1 and 2 and nCan f 3, respectively, as immunogens. Polyclonal anti-Can f 4 antiserum was pooled from 5 mice immunized 3 times 6 weeks apart with 25 µg of rCan f 4 and rCan f 6, respectively, by using aluminum hydroxide as an adjuvant. The antiserum was passed over 1-mL HiTrap NHS-activated columns (GE Healthcare Life Sciences, Uppsala, Sweden) with linked rCan f 4 and 6, respectively, and washed with 20 column volumes of PBS, followed by elution with 0.1 mol/L glycine-HCL, pH 2.5. Pooled antibodies were pH adjusted with 1 mol/L carbonate buffer, pH 9.6.

REFERENCES

- E1. Nilsson OB, Neimert-Andersson T, Bronge M, Grundstrom J, Sarma R, Uchtenhagen H, et al. Designing a multimer allergen for diagnosis and immunotherapy of dog allergic patients. *PLoS One* 2014;9:e111041.
- E2. Lebecque S, Dolecek C, Laffer S, Visco V, Denepoux S, Pin JJ, et al. Immunologic characterization of monoclonal antibodies that modulate human IgE binding to the major birch pollen allergen Bet v 1. *J Allergy Clin Immunol* 1997;99:374-84.

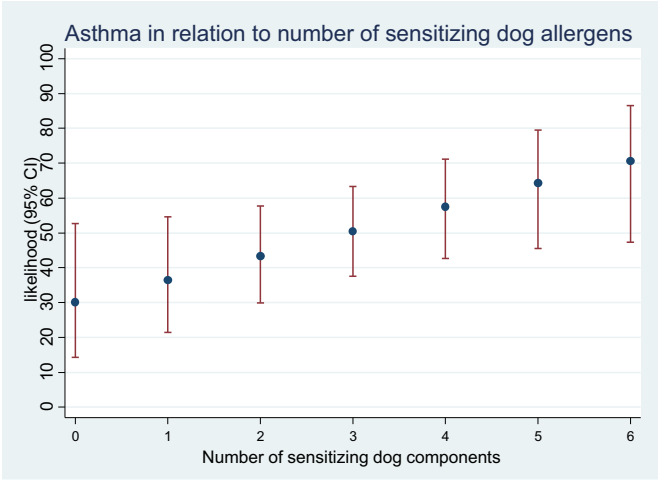


FIG E1. Likelihood (*y-axis*, percentage) of reporting asthma caused by exposure to dogs in relation to the number of IgE-positive dog allergen components (*x-axis*). Analysis included 60 patients sensitized to dog dander. *P* for trend = .038.

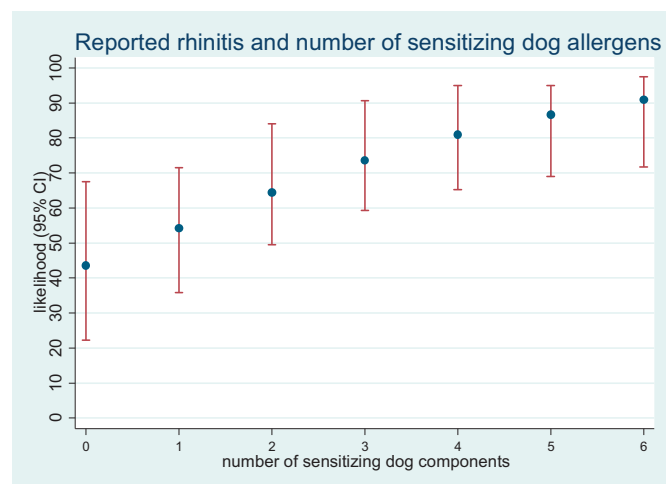


FIG E2. Likelihood (*y-axis*, percentage) of reporting dog-induced rhinitis in relation to the number of IgE-positive dog allergen components (*x-axis*). Analysis included 60 patients sensitized to dog dander. *P* for trend = .0067.

TABLE E1. Content of specific allergens in the dog dander extract used for NPTs

Specific component	Content of specific allergen in dog dander extract (ng/mL)
Can f 1	256
Can f 2	10
Can f 3	923
Can f 4	282
Can f 5	255
Can f 6	8

TABLE E2. Frequencies of reported symptoms in relation to sensitization to specific dog-derived components

Sensitizing allergen IgE >0.1 kU/L	Reported rhinitis, sensitized, no. (%)	Reported rhinitis, not sensitized, no. (%)	<i>P</i> value	Reported asthma, sensitized, no. (%)	Reported asthma, not sensitized, no. (%)	<i>P</i> value
Can f 1 (n = 39)	29/39 (74)	12/21 (57)	.17	21/39 (54)	9/21 (43)	.42
Can f 2 (n = 28)	22/28 (79)	19/32 (59)	.11	17/28 (61)	13/32 (41)	.12
Can f 3 (n = 16)	15/16 (94)	26/44 (59)	.012	11/16 (69)	19/44 (43)	.08
Can f 4 (n = 29)	23/29 (79)	18/31 (58)	.077	16/29 (55)	14/31 (45)	.44
Can f 5 (n = 37)	29/37 (78)	12/23 (52)	.034	22/37 (59)	8/23 (35)	.063
Can f 6 (n = 28)	24/28 (86)	17/32 (53)	.007	18/28 (64)	12/32 (38)	.038

Boldface indicates $P < .05$.

TABLE E3. ORs for reported rhinitis among patients sensitized to the different protein groups

Sensitized to IgE >0.1 kU/L	OR for reported dog-induced rhinitis	95% CI
Lipocalins	3.18	0.85-11.9
Serum albumin	6.66	0.75-59.3
Prostatic kallikrein	2.36	0.70-7.99

Adjusted for concomitant sensitization to other protein groups.

TABLE E4. ORs for reported asthma among patients sensitized to the different protein groups

Sensitized to IgE >0.1 kU/L	OR for reported dog-induced asthma	95% CI
Lipocalins	1.65	0.45-6.02
Serum albumin	2.02	0.55-7.46
Prostatic kallikrein	2.18	0.70-6.81

Adjusted for concomitant sensitization to other protein groups.

TABLE E5. Sensitization to common combinations of allergen protein groups

Sensitized to IgE >0.1 kU/L	OR for reported dog-induced rhinitis	95% CI
No components	0.07	0.01-0.65
Lipocalins only	0.61	0.18-2.06
Lipocalins and kallikrein	1.55	0.43-5.65
All protein groups	8.36	1.00-69.5
Kallikrein only	0.58	0.12-2.88

ORs for reported dog-induced rhinitis among patients sensitized to 0, 1, 2, or 3 different protein groups. Boldface indicates $P < .05$.

TABLE E6. Sensitization to common combinations of allergen protein groups

Sensitized to IgE >0.1 kU/L	OR for reported dog-induced asthma	95% CI
No components	0.17	0.02-1.58
Lipocalins only	0.58	0.18-1.91
Lipocalins and kallikrein	1.41	0.45-4.45
All protein groups	3.25	0.89-11.9
Kallikrein only	0.72	0.15-3.54

ORs for reported dog-induced asthma among patients sensitized to 0, 1, 2, or 3 different protein groups.