

Characterization of the dog lipocalin allergen Can f 6: the role in cross-reactivity with cat and horse

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To cite this article: Nilsson OB, Binnmyr J, Zoltowska A, Saarne T, van Hage M, Grönlund H. Characterization of the dog lipocalin allergen Can f 6: the role in cross-reactivity with cat and horse. *Allergy* 2012; DOI: 10.1111/j.1398-9995.2012.02826.x.

Keywords

allergen; Can f 6; cross-reactivity; dog allergy; dog dander.

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Accepted for publication 13 March 2012

DOI:10.1111/j.1398-9995.2012.02826.x

Edited by: Reto Crameri

Abstract

Background: Allergy to the domestic dog (*Canis familiaris*) affects 5–10% of the population in affluent countries. Three of four patients are allergic to more than one pet, which can only partially be explained by cross-reactivity between serum albumins. The lipocalin protein family harbours allergens in mammalian species.

Methods: We set out to clone and characterize a novel dog allergen, and investigate its potential role in cross-sensitization between dog, cat and horse. The gene encoding Can f 6 was amplified from dog skin and bladder cDNA libraries. The corresponding allergen was produced and purified by recombinant techniques and evaluated by SDS-PAGE, size exclusion chromatography, circular dichroism spectra, ELISA and basophil activation test.

Results: IgE antibodies to Can f 6 were found in serum from 38% of dog-sensitized subjects. Sequence similarities between the lipocalin allergens Can f 6, Fel d 4 (cat) and Equ c 1 (horse) suggested a probability for cross-reactivity, which was demonstrated by competitive ELISA. The biological relevance of Can f 6 was confirmed by basophil activation test in dog-allergic patients.

Conclusion: Can f 6 is a new lipocalin dog allergen that cross-reacts with lipocalins from horse and cat. Can f 6 and homologous allergens may contribute to multisensitization and symptoms in individuals allergic to mammals.

Sensitization and exposure to allergens from the domestic dog (*Canis familiaris*) is a frequent cause of allergic symptoms such as rhinitis and asthma (1). A common source of dog allergens is dander, which includes members of lipocalin protein family Can f 1, Can f 2 and Canf 4 (2–4) and dog albumin, Can f 3. Diagnostics as well as conventional immunotherapy of dog allergy is based on dander allergen extracts; however, available sources show great variability in the allergenic content (5). Can f 1 and Can f 2 have also been detected in dog saliva (6) and, recently, a major dog kallikrein allergen, Can f 5, (7) was found in dog urine, suggesting saliva and urine as important allergen sources (7, 8). A majority of mammalian allergens described as important sensitizers belong to the lipocalin protein family, including major allergens from dog, horse, cow, rat and mouse (9–11).

An overall similarity in allergen tertiary structure can serve as a basis for polysensitization. Thus, allergen-specific IgE antibodies may bind to similar epitopes across homologous allergens, which is here defined as cross-reactivity (12). The clinical importance of cross-reactivity has been established previously, as individual allergenic components can be used to simplify diagnostics of related allergies (13, 14). In addition, several studies have demonstrated a strong association between sensitization to dog, cat and horse among young children (15, 16), where the latter study also reported that monosensitization to horse was uncommon. This relationship might resemble cross-reactivity, as a great reduction in IgE reactivity in sera from cat-allergic individuals has been demonstrated after pre-incubation with dog dander (17).

In contrast to serum albumins (18, 19), the IgE-mediated cross-reactive capacity of lipocalins from different allergen sources is not clear. Although the sequence identity between some lipocalin allergens can be as low as ~20%, their structural resemblance is striking (20, 21). The overall similarity between lipocalin allergens may also explain cross-reactivity described,

Abbreviations

MW Molecular weight; CD-spectra Circular dichroism spectroscopy; aa amino acid.

including that between the major guinea-pig allergens Cav p 1 and Cav p 2 (22), the major horse allergen Equ c 1 and major mouse allergen Mus m 1 (23, 24) and between Can f 2 and the cat allergen Fel d 4 (21). While Can f 2 and Fel d 4 are cross-reactive despite sharing only 22% identity, it seems that a higher homology implies stronger cross-reactivity. However, not all lipocalins seem to cross-react (21, 23).

In conclusion, we present a new dog allergen, designated Can f 6 by the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-Committee. The allergen was identified in an effort to explore polysensitization within the group of patients allergic to pets. Can f 6 showed 38% IgE reactivity among dog-sensitized subjects and cross-reactivity with homologous components from cat (Fel d 4) and horse (Equ c 1), suggesting a new allergen that may lead to multisensitization and symptoms among patients allergic to mammals.

Materials and methods

Subjects

Sera from 100 patients (48 males and 52 females; range, 2–67 years; median, 24 years) with IgE responses ≥ 0.35 kU_A/l to dog dander extract (ImmunoCAP e5; Phadia, Uppsala, Sweden) were collected from the Department of Clinical Immunology and Transfusion Medicine at the Karolinska University Hospital, Solna.

Two patients with doctors' diagnosed allergy to dog, cat and horse and a positive ELISA test to recombinant (r)Can f 6, rFel d 4 and natural (n)Equ c 1 (21) were selected for basophil activation tests.

Cloning, production, purification and biochemical characterization

Details on cloning, production, purification and biochemical characterization of the allergens can be found in the supporting information.

Basophil activation test

Allergen-specific activation was assessed by monitoring the basophil activation markers CD203c and CD63 as described (25). For details, see the supporting information.

Quantification of IgE reactivity, ELISA competition experiments and measurement of cross-reactivity between rCan f 6, rFel d 4 and nEqu c 1

Quantitative ELISA measurements to rCan f 1, rCan f 2, nCan f 3, rCan f 6, rFel d 4 and nEqu c 1 were taken as described (21). IgE reactivity was considered positive if ≥ 0.1 kU_A/l. Sera from subjects with a positive IgE reactivity towards rCan f 6, rFel d 4 and nEqu c 1 were selected. Sera were diluted to 0.4 kU_A/l and incubated with 10-fold dilutions (1 : 2 v/v) of each respective lipocalin (rCan f 6, rFel d 4, nEqu c 1, rCan f 1, rCan f 2) or nCan f 3 as a control allergen overnight (ON) at 8°C. Plates were coated with 5 µg/ml of either rCan f 6, rFel d 4 or nEqu c 1 at 8°C ON and blocked for 1 h. Pre-incubated sera were added for 2 h, and IgE reactivity was measured.

Ethics statement

The study was approved by the local ethics committee at Karolinska Institutet. Written consent was obtained from the patients enrolled in the basophil trials.

Results

Amplification and production of Can f 6

The template gene for Can f 6 was identified by BLASTP search using template genes for Fel d 4 and Equ c 1 (26, 27). The resulting gene matched the translated mRNA (XP_855342) designated *Canis familiaris* similar to alpha-2u-globulin PGCL4. The sequence similarity and identity of Can f 6 to Fel d 4, Can f 6 to Equ c 1 and Fel d 4 to Equ c 1 were high, 80.0% and 67.4%, 72.5% and 55.1% and 79.5% and 64.2%, respectively (Fig. 1). An overall comparison of



Figure 1 Clustal alignment of five lipocalin sequences in this study. Percent identity to Can f 6 within brackets, Fel d 4 (67.4%),

Equ c 1 (55.1%), Can f 1 (22.9%) and Can f 2 (22.3%). Residues identical to Can f 6 are highlighted in blue.

Can f 6 with the most common lipocalin allergens with known sequences is presented in Table 1.

An open reading frame of 190 amino acids (aa) was identified, where the first 15 aa were predicted as a signal peptide by SignalP. The mature peptide revealed a predicted molecular weight (MW) of 20.193 kDa and an isoelectric point of 4.92. The gene encoding Can f 6 was amplified from both canine skin and bladder cDNA libraries, while no amplification could be obtained from tongue and submandibular gland libraries. Sequencing of the fragments resulted in sequences identical to the mRNA transcripts. In support, we identified nCan f 6 in a commercially available dog dander extract (kind gift of Enrique Fernandez-Caldas; Immunotek, Madrid) using an in-house-developed monoclonal antibody. The clone was previously determined not to cross-react with the dog lipocalin allergens rCan f 1, rCan f 2 or rCan f 4. Inhibition experiments with dog dander extract and rCan f 6 as reference revealed the presence of nCan f 6 at a concentration of 2 µg protein per ml aqueous extract (data not shown).

After adjustment of rare codons, 25 mg of soluble 6x-histidine-tagged rCan f 6 was expressed in *Escherichia coli* per litre culture medium. Purity of the final product was estimated by SDS-PAGE under reducing conditions to be >99% (Fig. S1A).

Characterization of rCan f 6

The observed molecular weight of purified rCan f 6 was compared to the homologous allergens rFel d 4 and nEqu c 1 using size exclusion chromatography. The proteins eluted as symmetrical peaks, where nEqu c 1 appears as an expected dimer and rCan f 6 and rFel d 4 as monomers (Fig. S1B). CD-spectra recorded for rCan f 6, rFel d 4 and nEqu c 1 revealed similar secondary structure elements, indicative of maintained fold (Fig. S2).

IgE binding of rCan f 6 and related allergens

The IgE reactivity of rCan f 6 was analysed in 100 subjects previously determined to be positive to dog extract

(>0.35 kU_A/l, median 2 kU_A/l, ImmunoCAP) (Fig. 2A and Table S1). The frequency of IgE binding to any of the three allergens rCan f 6, rFel d 4 and nEqu c 1 was 48%, and individually 38%, 35% and 42%, respectively. The score for the lipocalins rCan f 1 and rCan f 2 was 55% and 24% and for dog albumin, nCan f 3, 19%. The median IgE levels to rCan f 6, rFel d 4 and nEqu c 1 among all positive subjects were comparable, 1.7, 1.9 and 0.95 kU_A/l, respectively.

The majority were sensitized to all three allergens (Fig. 2B). Subjects sensitized to either rCan f 6, rFel d 4 or nEqu c 1 were 81.6%, 88.6% and 73.8% likely to be sensitized to all three allergens, respectively. The remaining subjects were either cosensitized with two of the three allergens, or monosensitized. Pairwise correlation of IgE levels for the three allergens was $r = 0.42$ for Can f 6 and Fel d 4, $r = 0.41$ for Can f 6 and Equ c 1 and $r = 0.42$ for Fel d 4 and Equ c 1, $P < 0.0001$ (Fig. 2C). The corresponding pairwise comparison of rCan f 6 with the unrelated allergen nCan f 3 did not correlate, $r = 0.06$, $P = 0.36$.

Biological assays

The biological relevance of Can f 6 was investigated in two dog-allergic individuals with IgE reactivity towards rCan f 6, rFel d 4 and nEqu c 1 by ELISA. The allergenicity of rCan f 6, rFel d 4 and nEqu c 1 was monitored by their capability to activate basophils upon allergen cross-linking of FcεRI-bound allergen-specific IgE (Fig. 3). The most potent activation was patient dependent, by either rCan f 6 or nEqu c 1, possibly reflecting the primary sensitization source.

Inhibition experiments

The potential cross-reactivity between rCan f 6, rFel d 4 and nEqu c 1 was investigated by inhibition ELISA. Serum from each subject was pre-incubated with titrated antigen and added to 96-well plates coated with respective allergen. Presentation of different manifestations from representative subjects is shown (Fig. 4).

The majority of subjects reacted with one or both of the two other allergens; however, two of fourteen subjects did

Table 1 Comparison of Can f 6 (175 aa) to common lipocalin allergens with known sequences among common mammalian species

Allergen	Animal	Amino acids	Similarity to Can f 6 (%)	Identity to Can f 6 (%)	UniProtKB accession#*
Bos d 2	Cow	156	53.5	23.9	Q28133
Can f 1	Dog	156	47.7	22.9	O18873
Can f 2	Dog	162	48.3	22.3	O18874
Can f 4	Dog	158	53.0	23.3	D7PBH4
Cav p 2	Guinea-pig	154	54.3	23.3	F0UZ11
Cav p 3	Guinea-pig	155	50.3	23.7	F0UZ12
Equ c 1	Horse	172	72.5	55.1	Q95182
Fel d 4	Cat	171	80.0	67.4	Q5VFH6
Fel d 7	Cat	154	42.9	21.7	E5D2Z5
Mus m 1	Mouse	162	78.7	43.4	P02762
Rat n 1	Rat	162	74.7	48.0	P02761

*<http://www.uniprot.org/>.

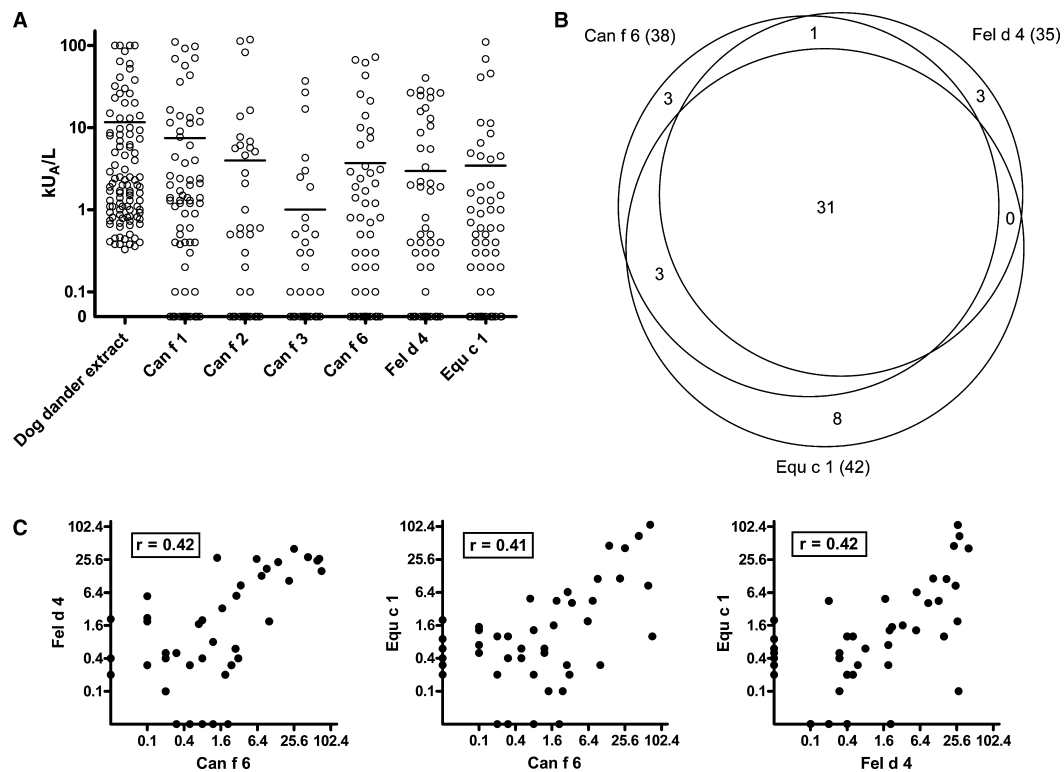


Figure 2 Analysis of IgE reactivity. (A) Responses to dog dander extract (ImmunoCAP e5), and the allergens rCan f 1, rCan f 2, nCan f 3, rCan f 6, rFel d 4 and nEqu c 1 (ELISA) among 100 dog-sensitized subjects with an IgE reactivity >0.35 kUA/l in ImmunoCAP.

Bars indicate mean values. (B) Cross-reactive overlap to rCan f 6, rFel d 4 and nEqu c 1 among 100 dog-sensitized individuals. (C) Comparison of binding to rCan f 6, rFel d 4 and nEqu c 1 (kUA/l) within the dog-sensitized population ($n = 100$).

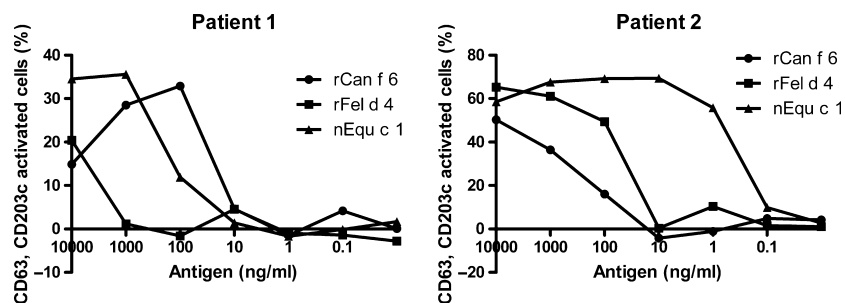


Figure 3 Basophil activation induced by serial dilutions of rCan f 6, rFel d 4 and nEqu c 1 (x-axis) in blood from two representative

dog-allergic patients. Positivity was defined as CD63- and CD203c-activated cells (y-axis) by flow cytometry.

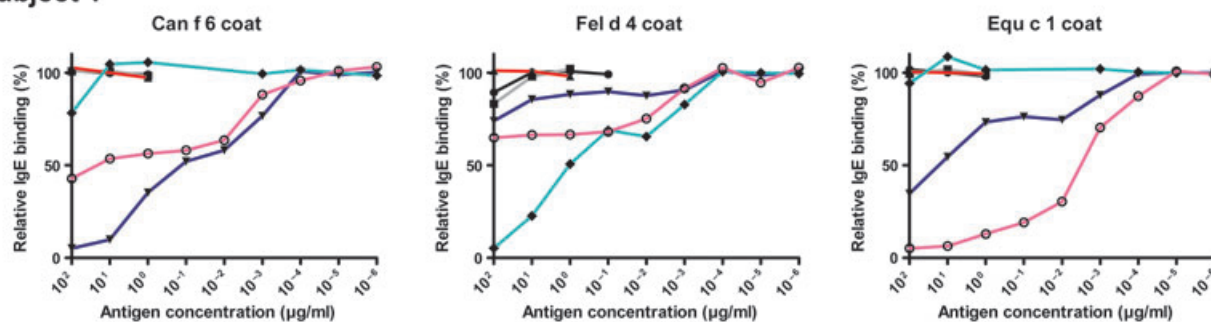
not show signs of cross-reactivity, even though they displayed IgE to all three antigens. Also, only at high concentrations of rCan f 1 and rCan f 2, a low inhibition could be detected in a few patients. We observed that IgE to nEqu c 1 appeared to be most common, followed by rCan f 6 and rFel d 4 (Fig. 4).

The degree of cross-reactivity varied between subjects. Homologous competition at the highest concentration reached the highest levels of inhibition ($>95\%$). At lower antigen concentrations, the most potent antigen in terms of inhibition levels varied, and cross-reactive antigens sometimes proved more efficient than the homologous allergen.

Discussion

In the present study, we describe a novel lipocalin allergen termed Can f 6. The allergen was amplified from canine skin and bladder cDNA libraries and detected in dog dander. Can f 6 was recognized by 38% of dog-sensitized subjects. Interestingly, close homologues are found in horse (Equ c 1) and cat (Fel d 4). Although lipocalins belong to a common allergen family among mammals, only a few cross-reactive allergen components have been described. Three other lipocalin allergens have been reported in dog; however, only Can f 2 has been shown to cross-react with Fel d 4, while cross-reactivity

Subject 1



Subject 2

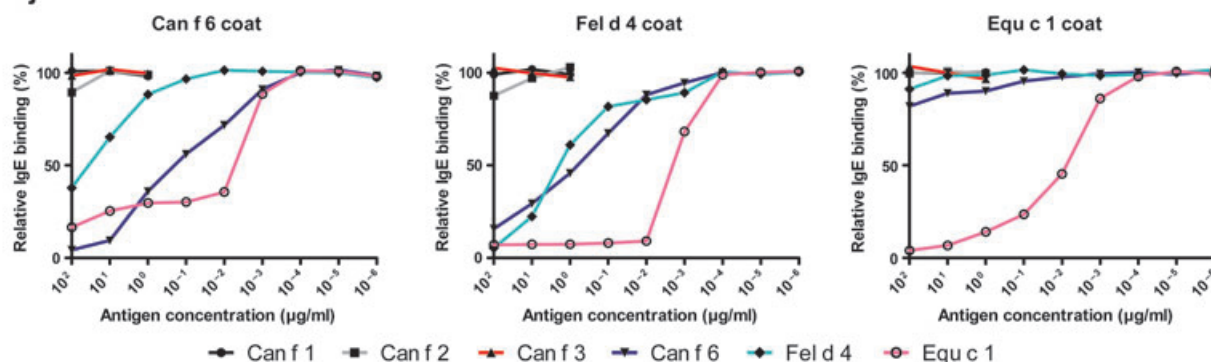


Figure 4 IgE-mediated cross-reactivity exhibited by rCan f 6, rFel d 4 and nEqu c 1, but not by rCan f 1, rCan f 2 or nCan f 3. Sera from two triple-sensitized individuals, subject 1: Can f 6 – 15.3 kUA/l, Fel d 4 – 7.0 kUA/l, Equ c 1 – 17.3 kUA/l and subject 2: Can f 6 – 9.2 kUA/l, Fel d 4 – 5.7 kUA/l, Equ c 1 – 13.8 kUA/l, were pre-

incubated with ten-fold dilutions (x-axis) of each allergen, followed by the detection of IgE responses by ELISA. Inhibition was calculated as response in percentage (%) of response to the coated allergen after the addition of antigen (y-axis).

to the major horse allergen Equ c 1 was absent (21). Compared to Can f 6, the dog lipocalin allergens Can f 1, 2 and 4 share a lower sequence identity with Fel d 4 and Equ c 1, which could explain the lack of cross-reactivity between these allergens. IgE antibodies to serum albumin have been suggested to cross-react with albumins from pet animals. The frequency of IgE binding to albumins was generally low (19%). However, the IgE binding to lipocalins was about twice as common (38%), suggesting that this protein family is an important factor in polysensitization among pet-allergic patients.

In a few subjects, there was a poor relationship between accumulated IgE reactivity to the individual allergen components and the commercial extract, investigated using sera from 100 dog-sensitized individuals. This indicates a low presence of the allergen in the extract and illustrates an advantage of component-resolved diagnostics (28).

We found that 38% of dog-sensitized subjects were positive to rCan f 6. This suggests that Can f 6 is more frequently recognized than the allergens Can f 2, Can f 3 and Can f 4, signifying Can f 6 as a key dog allergen. Importantly, we found several subjects with reactivity towards rCan f 6, who did not recognize rCan f 1 or rCan f 2. Moreover, quantification of IgE levels to rCan f 6 indicated that a significant fraction of total anti-canine IgE is directed towards this allergen (Table S1).

IgE antibody responses to rCan f 6 overlapped to a high extent with IgE binding to rFel d 4 and nEqu c 1 (Fig. 2B). We also found a good correlation of IgE reactivity between the three allergens (Fig. 2C), which in combination with the high sequence homology provides a basis for the cross-reactivity. Compliant with this suggestion, a previous study of cross-reactivity between lipocalins failed to show good inhibition unless the degree of homology was >50% (23). In support of the cross-reactive behaviour between the three lipocalins, no correlation was found with a member from another cross-reactive protein family, the dog serum albumin allergen nCan f 3.

We investigated the allergenic potential of rCan f 6, rFel d 4 and nEqu c 1 in a basophil activation test, using blood from dog-allergic patients. Specific activation of CD203c- and CD63-gated basophils was observed upon stimulation with all three allergens (Fig. 3). As expected, there was an allergen-dependent variation in potency between the individuals tested, suggesting allergenicity, primary sensitization source and structural relationship between these allergens.

Finally, we investigated IgE cross-reactivity by means of antigen-titration inhibition ELISA. There was a common, but variable cross-reactivity between these allergens. While some subjects exhibited IgE inhibition at antigen concentrations as low as 1 ng/ml, others showed little or no cross-reactivity.

Interestingly, the result illustrates how certain antibodies to related allergens are more specific at lower concentrations to cross-reactive components than to the homologous antigen (Fig 4). We speculate that the distribution of cross-reactive epitopes across the allergens determines the overall pattern of the inhibition curves, possibly explaining the enhanced specificity for some allergens at lower concentrations. These results point to the main sensitizing source, which could be valuable information prior to initiating allergen-specific immunotherapy. Based on sequence similarity and identity between Can f 6, Fel d 4 and Equ c 1, Can f 6 can be expected to cross-react with the major rodent allergens Mus m 1 (mouse) and Rat n 1 (rat), similarly as demonstrated for Equ c 1 (23). Also, we do not expect Can f 6 to cross-react with known human lipocalins, such as human tear lipocalin (TL), which shares 19.4% identity with Can f 6. In support of this assumption, no cross-reactivity was detected between Equ c 1 and human TL (22.7% identity), while cross-reactivity was demonstrated between TL and Can f 1 (56.3% identity) (23).

In conclusion, we here describe a novel dog allergen, Can f 6, with high sequence homology to similar lipocalin allergens in horse (Equ c 1) and cat (Fel d 4) and thus a possible candidate for cross-species sensitization. rCan f 6 was identified by nearly 40% of dog-sensitized subjects as a common allergen, providing an important contribution to the dog allergen family.

Acknowledgments

The authors would like to acknowledge Adnane Achour and Hannes Uchtenhagen at the Department of Microbiology, Tumor and Cell Biology (MTC) for assistance with the CD-spectra measurements.

Author contribution

OBN participated in all stages and experiments of the project and was the main author of the manuscript. OBN and HG designed the project; HG supervised the experiments and together with OBN wrote the manuscript. Other authors performed the experiments and provided critical review of the manuscript.

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Funding

This research was funded by grants from the Swedish Asthma and Allergy Association's Research Foundation, the Konsul Th C Bergh Foundation, the Cancer and Allergy Foundation, the Swedish Research Council, the Swedish Heart-Lung Foundation, the King Gustaf V 80th Birthday Foundation, the Hesselman Foundation, the Centre for Allergy Research and the Bernard Osher Initiative for Research on Severe Asthma at Karolinska Institutet, the Stockholm County Council, the Theme Center for Inflammatory Diseases and Karolinska Institutet.

Conflict of interest

The authors do not have any conflict of interest to declare.

Supporting Information

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

Figure S1. Biochemical analysis of rCan f 6. (A) SDS-PAGE of purified dog lipocalin allergens under reducing conditions with Coomassie blue staining. MW markers (lanes 1,7, MW (kDa)), rCan f 1 (lane 2), rCan f 2 (lane 3), rCan f 6 (lane 4), rFel d 4 (lane 5) and nEqu c 1 (lane 6). (B) Analytical size exclusion chromatography of rCan f 6, rFel d 4 and nEqu c 1. The dots denote bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and Ribonuclease A (13.7 kDa) as molecular weight markers.

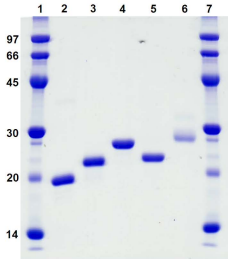
Figure S2. Far-UV CD analysis of rCan f 6, rFel d 4 and nEqu c 1. The spectra are expressed as mean residue ellipticities (θ) at a given wavelength.

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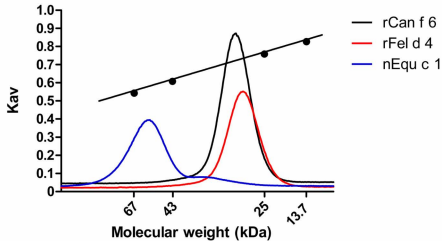
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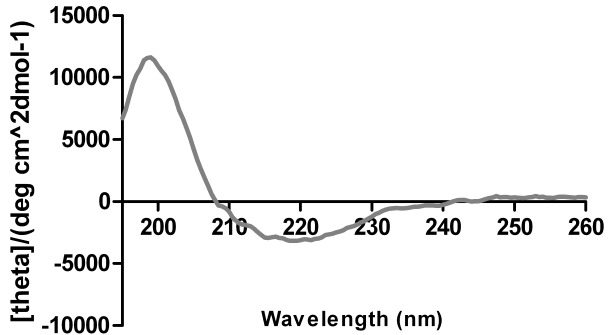
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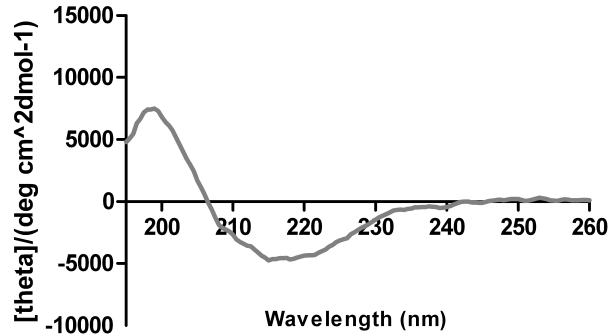
B



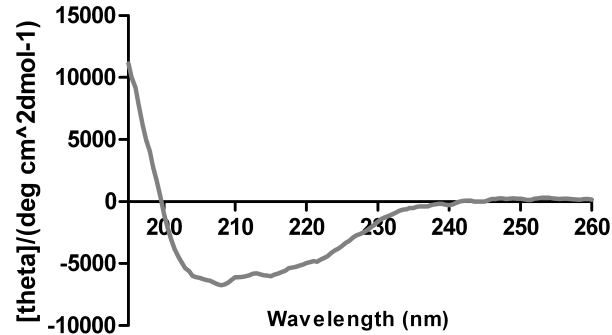
rCan f 6



rFel d 4



nEqu c 1



Characterization of the dog lipocalin allergen Can f 6: the role in cross-reactivity with cat and horse

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Supporting information

Materials and Methods

Cloning, production and purification of allergens- cDNA libraries from skin, tongue, bladder (Zyagen, San Diego, CA, USA) and submandibular gland (Open Biosystems, Huntsville, AL, USA) tissues from canine were screened for the Can f 6 gene. Amplification was carried out using 5' caccaggaagaaaacgatgttg and 3' ctcagcactggagacc primers, aligning to the gene corresponding to Can f 6 (XM_850249). Sequencing was done using PCR-products as templates (BigDye Terminator v1.1 Cycle Sequencing Kit and ABI PRISM 310 Genetic Analyzer, Applied Biosystems, CA, USA).

For the purpose of expressing rCan f 6, the mature coding sequence was identified by extracting the 15 amino acid signal sequence using SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) from the complete open reading frame (XP_855342). The reverse-translated DNA sequence suited for expression in *Escherichia coli*

(*E. coli*) was purchased from Geneart (Regensburg, Germany). The resulting PCR-fragment was subcloned into the vector pET20b (Novagen, EMD Chemicals Inc., Darmstadt, Germany) using the NdeI and XhoI restriction sites, and expressed in *E. coli* BL21(DE3)pLysS (Novagen), resulting in a C-terminally 6x-histidine-tagged protein. Production and purification of rCan f 6, and the other allergens used for the study, rCan f 1, rCan f 2, rFel d 4, nCan f 3 and nEqu c 1 was carried out as previously described (1).

Identification of nCan f 6 in a commercially available dog dander extract- Monoclonal antibodies (mAb) specific for Can f 6 was produced as follows. Female BALB/c mice were immunized with 3 times 10 µg rCan f 6 (2). Animal experiments was approved by the Swedish local ethics committee for animal welfare. Isolation and culture of single antibody secreting specific for rCan f 6 was performed essentially as described (3). First, a sensitive range of the antibody towards rCan f 6 suitable for antigen competition ELISA was investigated by titration (2). Secondly, the binding of the mAb was tested for binding to the dog lipocalin allergens rCan f 1, rCan f 2 and rCan f 4. There was no cross-reactivity observed to rCan f 1, rCan f 2 or rCan f 4 ($>10^6$ fold). Natural Can f 6 was detected in a commercial dog dander extract (Immunotech, Madrid, Spain). Three fold dilutions of the extract or of rCan f 6 (100-0.01 µg/ml) used as reference were mixed 1:2 with the preset dilution (1:100), of the antibody and the inhibition was investigated as previously described (1).

Characterization of rCan f 6- The molecular weight of rCan f 6 was estimated by SDS-PAGE under reducing conditions as described previously (4). Circular dichroism (CD) spectroscopy experiments for all allergens were done at 20°C in 20 mM KH₂PO₄/K₂HPO₄ (pH 7.5) using 0.12 mg/ml of each purified protein. The spectrum was recorded in a 1 mm pathlength cell

with a JASCO spectrometer (JASCO, Mary's Court, Easton, MD, USA) equipped with a thermoelectric temperature controller.

Basophil activation test- Allergen-specific activation was assessed by monitoring the basophil activation markers CD203c and CD63 as described (5). Briefly, 10-fold serial dilutions of either rCan f 6 or rFel d 4 (10 µg/ml to 10⁻⁵ µg/ml final concentration), medium (negative control), 1 µg/ml rabbit anti-human IgE and BCCR STCON (positive controls, kind gift from Phadia and Skafte medlab, Onsala, Sweden, respectively) were added to venous blood samples from two dog, cat and horse-sensitized individuals. The samples were further incubated with anti-CD63 and anti-CD203c monoclonal antibodies (Beckman Coulter, Brea CA, USA) and analyzed by flow cytometry using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data analysis was performed using FlowJo (Treestar, Ashland, Or, USA). Basophils were identified by gating for CD203c positive cells and the magnitude of allergen-activation was calculated as the percentage of upregulated CD63 and CD203c cells among the gated basophils.

Figure legends

Figure S1. Biochemical analysis of rCan f 6. (A) SDS-PAGE of purified dog lipocalin allergens under reducing conditions with comassie blue staining. MW markers (lane 1,7, MW (kDa)), rCan f 1 (lane 2), rCan f 2 (lane 3), rCan f 6 (lane 4), rFel d 4 (lane 5) and nEqu c 1 (lane 6). (B) Analytical size exclusion chromatography of rCan f 6, rFel d 4 and nEqu c 1. The dots denote bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and Ribonuclease A (13.7 kDa) as molecular weight markers.

Figure S2. Far-UV CD analysis of rCan f 6, rFel d 4 and nEqu c 1. The spectra are expressed as mean residue ellipticities (θ) at a given wavelength.

Figures

Figure S1.

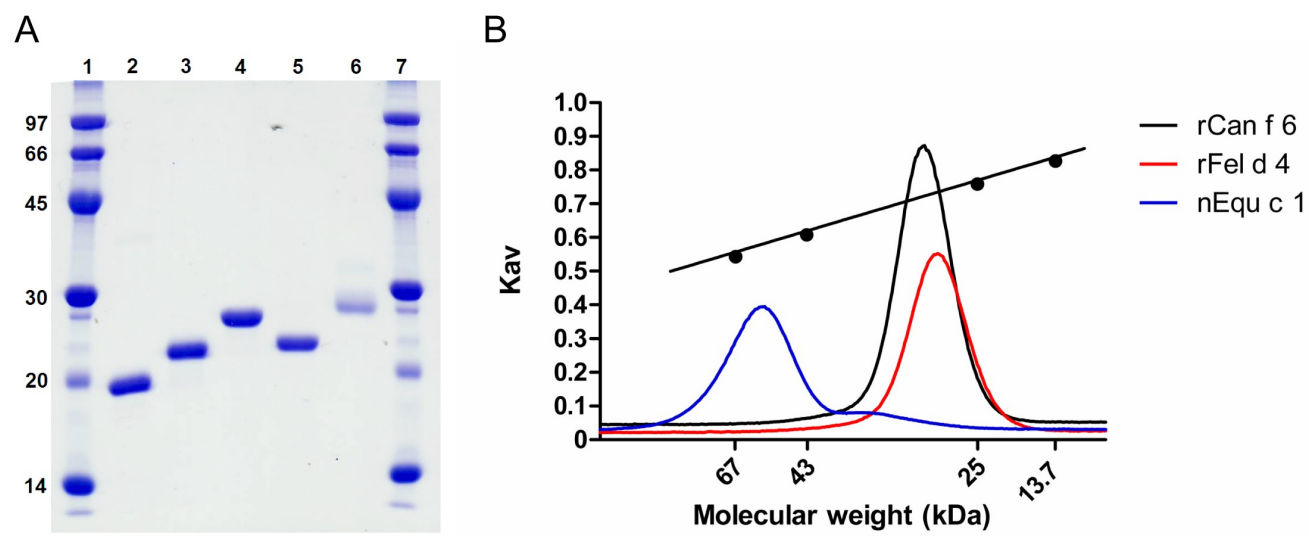


Figure S2

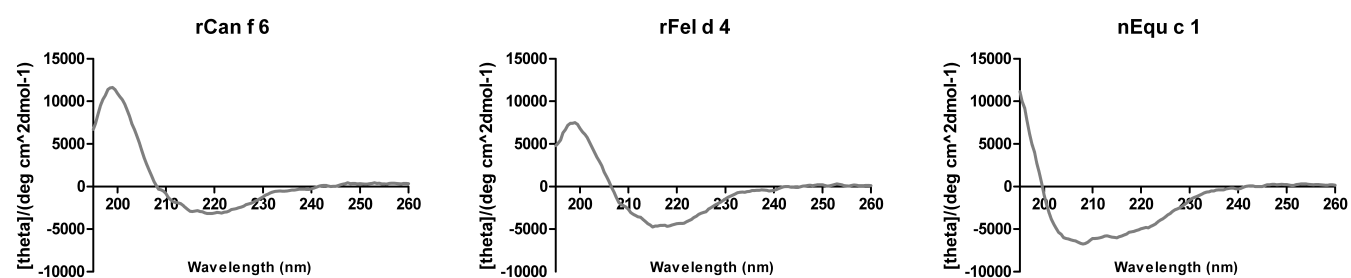


Table S1. Dog sensitized subjects and tested allergens.

Subject	Dog dander	Can f 1	Can f 2	Can f 3	Can f 6	Fel d 4	Equ c 1	Subject	Dog dander	Can f 1	Can f 2	Can f 3	Can f 6	Fel d 4	Equ c 1
1	6,9	4,4	4,6	2,5	3,4	8,7	4,1	51	8	6,1	0	0	0	0	0,2
2	64	68,9	0,3	0,1	43,2	28,4	68,6	52	100	110	118	0	72,3	15,8	1
3	0,81	0,38	0	0	0	0	0	53	4,9	1,3	0	0	0,1	2,2	1,5
4	0,83	0	0,1	0	0	0	0	54	26	11,3	5,1	0,4	3,1	0,4	0,2
5	0,45	0	0	0	0	0	0	55	1,6	0	0	0	0	2,1	0
6	1,3	0,6	0	0	0,8	0,4	0,2	56	1,1	0	0	0	0	0	0
7	1,7	2,1	2,1	1,9	1,2	0,8	0,6	57	1,6	1,2	0	0	0	0	0
8	2	2	0	0	0,8	0	0	58	0,5	0	0	0	0	0	0
9	14	0	0	0	0	0	0	59	0,79	0,2	0	0	0	0	0
10	0,44	0	0	0	0	0	0	60	1,7	0	0	0	0	0	0
11	10	0	0	0	14	23,1	45,5	61	1,3	0	0	0	0	0	0
12	1	0,1	0,1	0	0	0	0	62	2,5	2,4	0	0	0	0	2
13	0,45	0,1	0	0	0	0	0	63	1,6	13,9	16,3	16,9	9,1	17,4	11,3
14	1,4	0	0	0	0,1	0,3	0,5	64	6,2	2,6	0	0	2,1	0	0
15	85	70,4	0	0	0	0	0,9	65	41	0	0	0	0	0	0
16	15	13,2	5,6	4,3	7,5	12,9	4,5	66	2,3	1,4	0	0	0	0	0
17	9,7	0	0	0	0	0	0,3	67	5	1,4	0	0	0,7	1,7	4,9
18	0,38	0	0	0	0	0	0	68	0,92	0	0	0	0	0	0
19	1	0,9	0	0	0	0	0	69	1,9	1,4	0	0	0	0	0
20	0,4	0	0	0	0,3	0	0,4	70	1,1	0	0	0	0	0	0
21	60	56,8	13,7	0	66,7	26,6	110	71	0,87	0	0	0	0	0	0
22	52	0	0	0,1	21,2	10,5	11,5	72	1,1	1,2	0	0	0,5	0,3	0,4
23	2,5	1,7	0	0	1,9	0,2	4,5	73	8,2	9	0,6	0,6	0,1	5,5	1,3
24	32	36,1	0	0	0	0	0,6	74	23	11,5	0,2	0,3	6,2	26,4	1,9
25	5,8	4	0	0	0	0	0,3	75	0,81	2,3	2,8	3	1,7	3,3	1,6
26	100	0,5	0,5	37	0,8	2	1,3	76	9,3	16,4	0	0	0	0	0
27	0,38	0	0	0	0	0	0	77	7,3	0	7,7	0	0	0,2	0
28	5,9	3,7	0	0	0	0	0,4	78	1,9	0	0	0	0	0	0
29	2,5	0	0	0	2,9	5,6	6,5	79	26	7,7	0	0	1,2	0	0,5
30	1,5	0,1	0	0	2,8	0,6	0,3	80	1,2	0	0	0	0	0	0
31	0,36	0	0	0	0	0	0	81	2,4	0	0	0	0,1	1,9	0,7
32	2,3	1,5	0	0	0,5	0	0,6	82	3,5	0	1	0	0	0	0
33	13	0,4	0	0	0	0	0	83	1,1	0	0	0	0	0	0
34	0,46	0	0	0	0	0	0	84	100	91,8	113	26,9	61,8	24,6	8,5
35	1,7	1,2	0	0	0	0	0	85	0,93	0	0	0	0	0	0
36	0,82	0	0	0	0	0	0	86	0,65	0	0	0,2	0	0	0
37	0,67	0	0	0	0	0	0	87	1,3	0,4	0	0	0	0	0
38	3,1	0,4	0,6	0	0,3	0	0	88	20	2,4	6,1	0	0	0	0
39	2,1	1,3	0	0	0	0	0	89	0,41	0	0	0	0	0	0
40	1	0,9	0	0	0	0	0	90	2,9	1,3	0	0	0,2	0,5	0,2
41	0,38	0	0	0	0	0	0	91	100	97,5	82,4	0,5	25,5	40,3	41
42	4	0,3	0,5	0	0	0	0	92	13	0,6	5,7	0	2,4	0,3	0,1
43	0,69	0,4	0,6	0,5	0,3	0,5	1	93	30	43,5	0	0	1,4	27,6	0,1
44	0,62	0	0	0	0	0	0	94	8,6	2	0,5	0,3	0,2	0,4	1
45	1,1	0	0	0	0,2	0,1	0	95	20	0	0	0	0	0	0
46	2	0	0	0,1	0	0	0	96	0,33	0	0	0	0	0	0
47	0,77	0	0	0	0	0	0	97	0,73	0	0	0,1	0	0	0
48	2	1,1	0	0,8	0	0	0	98	0,74	0	0	0	0	0	0
49	0,67	0	0	0	0	0,4	0	99	4,6	0	0	0	0	0	0
50	8,3	11,8	0	0	0	0	0,3	100	38	16,2	6,8	0	10	1,9	0,3

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Table 1 Future directions for endotyping of asthma

Pathogenic mechanism	Proposed biomarker	Level of evidence			References
		Intervention trials (I)	Association studies (A)	Experimental (in vitro data/animal models) (E)	
Eosinophilic inflammation	IL-5	+	+	+	I:1-7 A:8,9 E:10-13 E: 14,15 A: 16-25
	IL-5/STAT-6/ Notch Eotaxin	-	-	+	E: 26-28 A: 29-33
	RANTES	-	+	+	E:28,34 A:35
	GM-CSF	-	+	+	E:36 E:37,38 A: 39,40
	CD48	-	-	+	E:41-48 E:49-52 A:53,54
	IL-33	-	+	+	E:55-58 E:59,60 A, E:61 E:62,63 A:64
	IL-23	-	-	+	E:65 A:66,67
	Proangiogenic bone marrow progenitors	-	+	+	E:68,69 E:70-72 E:73 E:74
	IL-9	-	-	+	
	IL-7	-	+	+	
	Leptin	-	-	+	
	Osteopontin	-	+	+	
	Galectin-9	-	+	+	
	Lactoferrin	-	-	+	
	Tyrosine kinase	-	-	+	
	TSLP	-	-	+	
Th2	IL-13/Periostin				I:75-78
profile/allergic inflammation		+	+	+	A:79-84 E:85-90 I:78, 91 A:80-84, 92
	IL-4	+	+	+	

	IL-9				E:88,90,93 I:94
		+	+	+	A:95,96
	CCR4, CCR8	-	+	+	E:97,98 A:99
	TSLP	?	+	+	E:100 A:101-104
	TARC	-	+	+	E:105-118 A:119-122
	IL-25 CRTH2 and DP1	-	-	+	E:123 E:124-128 A:129-132
	receptors ICOS/ICOS-L IL-31	?	+	+	E:133-144 E:145-149 A:150
		-	+	+	
	IL-33 and T1/ST-2	-	+	+	E:151,152 A:153-156
	IL-19	-	+	+	E:157-167 A:168
	IL-22	-	-	+	E:169,170 E:171
	Histamine 4 receptor	-	-	+	E:172
	NKT cells	-	+	+	A:173,174
					E:175,176
Intrinsic/non-atopic asthma	IL-18	-	+	+	A:177-179
	IL-15	-	+	+	E:180-184 A:185,186
	IP-10	-	+	+	E:187 A:188,189
	IFN-gamma Allergen specific Th1	-	-	+	E:190 E:190,191 E:192
	cells CD26	-	-	+	E:193
Neutrophilic inflammation	IL-8	-	+	+	A:194-198
	IL-17A	-	+	+	E:199,200 A:201
	LTB4	-	-	+	E:202 E:203

	IL-12/IL-18	-	-	+	E:199
	IL-32	-	-	-	E:204
	PAMS, DAMPS, SDF	-	-	-	E:200
	BDNF; MIP-3a/CCL-20;	-	+	-	A:205
	IL-1 b				
Neurogenic	NGF/TrkA/p75				A:206-212
		-	+	+	
inflammation					E:212-221
	BDNF	-	+	+	A:210-212, 222-227
					E:217-219, 221
	NT-3, NT-4	-	+	+	A:210-12
					E:221
	Tachykinins	+/-	+	-	I:228
					A:229,230
	VIP/VPAC1, VPAC2	-	-	+	E:231,232
	CGRP/RAMP1	-	+	+	A:233
					E:234
	Kidins220/ARMS	-	-	+	E:235
	Neurturin	-	-	+	E:236
	Semaphorins	-	-	+	E:237
Immune	IL-10				A:238,239
		-	+	+	
regulation in					E:240-245
asthma	T regulatory cells	-	+	+	A:246
					E:247,248
	IL-27	-	-	+	E:249
	CTLA4	-	-	+	E:250
	PD-1	-	-	+	E:251
	pDC	-	-	+	E:252
	TGF-beta	-	-	+	E:253
	type I IFN	-	-	+	E:254
	TNFRSF25	-	-	+	E:255
	osteopontin	-	-	+	E:256-258
	IDO	-	+	+	A:259,260
					E:261
	vitamin D	-	+	-	A:262-265
Innate	TNF- α				I:266-270
		+	+	+	A:270,271
immunity in					E:272-276
asthma					A:277-279
	IFNs	?	+	+	
					E:279-286
	IL-15	-	+/-	+	A:287, 288
					E:288, 289
	TLRs	-	+	+	A:290-294

	NK cells	-	-	+	E:286, 295-302 E:303
	Purinergic inflammation	-	+	+	A:304
	PAR activated receptors	-	-	+	E:305-311 E:312-318
	Chitinase-like proteins	-	+	+	A: 319-325
	Complement/C5L2	+	-	+	E:317, 326-328 I:329
	APRIL	-	-	+	E:330-334 E:335
	MBL	-	+	-	A:336-338
	Defensins	-	+	+	A:339
	Collectins	-	-	+	E:340 E:341,342
	Cathelidicin	-	-	+	E:343
	Granzyme	-	+	+	A:344-348
	IRAK-M	-	-	+	E:348-350 E:351
	Nuocytes	-	-	+	E:352
	Innate helper type 2 cells	-	-	+	E:353
Resolution of	Lipoxins	-	+	+	A:354-360
inflammation	Resolvins	-	-	+	E:361-364 E:365-368
	Protectins	-	+	+	A, E: 369
	Maresins	-	-	+	E:370
Lung	Sputum/BAL eosinophils				I:6; 371
remodeling		+/-	+	+/-	A:372-374
	MMP,TIMP	?	+	+	E:375-379 A:380-388
	ADAM 33, ADAMTS	-	+	+	E:384, 389-396 A:397-402
	IL-13/IL-4	-	+	+	E:403 A:404
	CC and CXC	-	-	+	E:405-407 E:408-410
	chemokines	-	+	+	A:411-414
	VEGF, angiopoietins 1	-	+	+	E:415-419
	and 2	-	+	-	A:420
	Tumstatin	-	+	-	A:421
	Retinoid receptors	-	+	+	A:422
	Epidermal growth	-	+	+	

	factors				E:423-425
	Amphiregulin	-	+	-	A:425
	Periostin	-	-	+	E:426
	Fibulin	-	-	+	E:427
	Decorin	-	-	+	E:428
	Oncostatin M	-	-	+	E:429
	LIGHT	-	-	+	E:430
	Relaxin	-	-	+	E:431
	Endothelin-1				A:432-435
		-	+	+	
	Tight junctions				E:436-439
		-	+	+	A:440, 441
	Airway fibroblasts and				E:442
	epithelial cells				E:443-445
	Osteopontin	-	-	+	
Airway	IL-13/IL-4	-	-	+	E:446,447
	NKT cells	-	-	+	E:406,407,448, 449
hyperreactivity	TLR-7	-	-	+	E:449-453
	RhoA /miR-133a	-	-	+	E:454
		-	-	+	E: 455,456
	balance				
	ICOS-L	-	-	+	E:457
Sex-related	Estrogen's and Th2				E:458-461
differences in	responses and	-	-	+	
asthma	inflammation in adults				
	Estrogens and lung	-	-	+/-	E: 461, 452
	remodeling				
	HIF-1a	-	-	+	E:463
	DHEA and immune	-	-	+	E:464
	regulation				
Age-related	Development and				A:465
differences in	function of regulatory T	-	+	-	
asthma	cells				
	Eosinophil function	-	+	-	A:466
Susceptibility	Eosinophilic markers				I:467-469
to asthma	(sputum eosinophils and	+	+	-	A:470-473
exacerbations	exhaled NO)				
	IL-8 and neutrophils	-	+	+	A:474-477
					E:478-479
	Total IgE				I:480-484
		+	+	-	
	FCER2 T2206C variant	-	+	-	A:485,486
					A:487

	Vitamin D	?	+	-	A:488, 489
	Urinary bromotyrosine	-	+	-	A:490
	IL-4 receptor- α	-	+	-	A:491
	iNOS pathway	-	-	+	E:492
	Urinary LTE4	-	+	-	A:493
Severe asthma	Eosinophilic				A:494-498
		-	+	-	
	inflammation				
	NO and related enzymes	-	+	-	A:499
	EDN	-	+	-	A:500
	Eosinophil protein in				A:501
		-	+	-	
	airway macrophages				
	IL-13	-	+	-	A:502
	IL-4	-	+	-	A:503,504
	OX40L	-	+	-	A:504,505
	TSLP	-	+	-	A:506
	Fc ϵ RI	-	+	-	A:503,507
	Mast cell infiltration	-	+	-	A:495, 508,509
	Reduced PGE(2) to				A:510
		-	+	-	
	cysLT ratio				
	Neutrophilic				A:494, 511-514
		-	+	-	
	inflammation				
	IL-17	-	+	-	A:515
	BDNF	-	+	-	A:516
	Osteopontin	-	+	-	A:517,518
	Extracellular cyclophilin	-	+	-	A:519
	CREB	-	+	-	A:520
	RIP-2 (receptor-				A:521
		-	+	-	
	interacting serine-				
		-	+	-	
	threonine kinase 2)				
	Vitamin D	-	+	-	A:522
Steroid	Neutrophilic				A:479,523,524
			+	-	
resistance	inflammation				
	HDAC recruitment	-	+/-	+	A:525, 526
					E: 527
	GR-beta	-	+/-	+	A:525, 526
					E:527, 528
	IL-17	-	-	+	E:529-531
	IL-27/IFN- γ	-	-	+	E:532

Table legend:

I = references for interventional trials

A = references for association trials

E = references for experimental models

? = ongoing targeted interventions with no data available yet

+/- = controversial or weak evidence

+ = strong/moderate supportive evidence

- = no evidence

References Table 1

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