Supplementary data for the article:

Apostolovic, D.; Sánchez-Vidaurre, S.; Waden, K.; Curin, M.; Grundström, J.; Gafvelin, G.; Cirkovic Velickovic, T.; Grönlund, H.; Thomas, W. R.; Valenta, R.; et al. The Cat Lipocalin Fel d 7 and Its Cross-Reactivity with the Dog Lipocalin Can f 1. *Allergy: European Journal of Allergy and Clinical Immunology* **2016**, *71* (10), 1490–1495. https://doi.org/10.1111/all.12955

Supporting information

The cat lipocalin Fel d 7 and its cross-reactivity with the dog lipocalin Can f 1

D. Apostolovic[1,2*], S. Sánchez-Vidaurre[1*], K. Waden[1*], M. Curin[3], J. Grundström[1], G. Gafvelin[4], T. Cirkovic Velickovic[2], H. Grönlund[4], WR. Thomas[5], R. Valenta[3], C. Hamsten[±1,6] & M. van Hage[±1]

[1]Department of Medicine Solna, Immunology and Allergy Unit, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden;

[2]Center of Excellence for Molecular Food Sciences, Faculty of Chemistry, University of Belgrade, Belgrade, Serbia;

[3]Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Austria;

[4]Department of Clinical Neuroscience, Therapeutic Immune Design Unit, Center for Molecular Medicine (CMM), Karolinska Institutet, Stockholm, Sweden;

[5]Center for Child Health Research, Telethon Institute of Child Health Research, University of Western Australia, Perth, Australia;

[6]Center for Inflammatory Diseases, Karolinska Institutet, Stockholm, Sweden.

[*] These authors contributed equally to this work.

[‡] These authors contributed equally to this work.

Correspondence

Marianne van Hage, MD, PhD

Karolinska Institutet,

Department of Medicine, Solna

Immunology and Allergy Unit

Karolinska University Hospital Solna L2:04

SE - 171 76 Stockholm, Sweden

Tel +46-8-517 75942, Fax +46-8-33 57 24

E-mail: marianne.van.hage@ki.se

Methods

Allergen preparations

BL21 Star (DE3) *Escherichia coli* (Invitrogen, Carlsbad, CA) were transformed with the p80QE plasmid containing the gene for Fel d 7 (1). Cells were grown for four hours at 37°C and protein expression was induced by addition of isopropyl-b-thiogalactopyranoside (IPTG) to a final concentration of 0.5 M for three hours and the cells were subsequently pelleted (16 800 g, 10min). After sonication (Soniprep 150 ultrasonic disintegrator, Sanyo Gallenkamp, Uxbridge, UK) by 10 sec (10ma) bursts and centrifugation (16 800 g, 10 min), the soluble supernatant fraction was sterile filtered and purified twice on 6-His-tag affinity IMAC Ni^{2+} column (GE healthcare, Uppsala, Sweden) using an ÄKTA purifier (GE Healthcare) followed by Amicon Ultra 15 (Merck Millipore, Darmstadt, Germany) filtration in the range from 3 to 30 kDa range.

Recombinant (r) Can f 1 protein was prepared as previously described (2). Briefly, 6-His-tagged Can f 1 was produced by BL21 (DE3) pLysS (Novagen, EMD Chemicals, Darmstadt, Germany) *E.coli* transformed with pET20b (Novagen) containing the gene for Can f 1. Protein concentrations were determined by BCA™ protein assay (Pierce, Rockford, IL).

Circular dichroism spectroscopy

Circular dichroism (CD)-spectra were recorded on a JASCO J-815 spectropolarimeter (JASCO, Tokyo, Japan). Samples (0.5 mg/ml of rFel d 7 and rCan f 1 in PBS, pH 7.4) were analyzed at 25°C in a 0.1 mm path length quartz cell for far-UV and a 10 mm path length quartz cell for near-UV spectra. The spectra were collected in 0.1 nm steps at a rate of 50 nm/min over the wavelength range 195-260 nm for far-UV and 260–320 nm for near-UV. Each spectrum was acquired five times and the results were averaged. Results were recorded

in mdeg and converted to molar Δε in M^{-1} cm^{-1} and mean residue ΔεMRV for near UV and far UV, respectively. The percentage of secondary structure motifs were determined in the CONTIN software with the SP37 protein set in CDPro.

IgE ELISA for Fel d 7 and Can f 1

IgE levels to rFel d 7 and rCan f 1 were determined by enzyme-linked immunosorbent assay (ELISA) (2, 3).Half-area microtiter plates (96 wells, Greiner bio-one, Frickenhausen, Germany) were coated overnight at 4°C with 0.5 µg of rFel d 7 or rCan f 1, followed by blocking (PBS containing 1% BSA and 0.5% Tween 20, pH 7.4) for 2 h at room temperature. Standard curves created with active human IgE (Abcam, Cambridge, UK) and undiluted serum samples were incubated for 2 h. Detection was performed with rabbit anti-human IgE antibody (1h, 1:2000; MIAB, Uppsala, Sweden) followed by alkaline phosphatase (AP) conjugated goat anti-rabbit antibody (1h, 1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA). Finally, p-nitrophenyl phosphate substrate (Sigma, St. Louis, MO) was added and incubated for 15 min. The absorbance was measured at 405 nm. Sera with an IgE antibody level >100 kU_A/l were re-run at 1:10 dilution. The IgE ELISAs were repeated with an inter-assay variation of 12%. IgE values to rFel d 7 were considered positive when the IgE level exceeded the mean of the 45 negative controls + 3 SD ($kU_A/l \geq 0.12$ to Fel d 7). IgE values to rCan f 1 were regarded as positive when the IgE level exceeded the previously established cut-off for rCan f 1 (≥ 0.1 kU_A/l) (3). Spearman's correlation test was used for comparing rFel d 7- and rCan f 1-specific IgE levels, where $p<0.05$ was considered significant. Analyses were performed using Graphpad Prism 5 software (Graphpad Software Inc., San Diego, CA).

Basophil activation test

Allergen-specific basophil degranulation was analyzed by monitoring the basophil activation markers CD203c and CD63, as previously described (4). Briefly, 10-fold serial dilutions of rFel d 7, rCan f 1 (10 μg/ml to 10^{-7} μg/ml) or two irrelevant control allergens (BSA from New England BioLabs, MA and rLep d 2.01, in house production), medium (negative control) and 1 μg/ml rabbit anti-human IgE (Phadia AB, Uppsala, Sweden) were added to heparinized venous blood samples from four patients with doctor's diagnosed allergy to cat and IgE positive to Fel d 7, whereof two were also co-sensitized to Can f 1. Serum from a non-atopic subject IgE negative to rFel d 7 (and Can f 1) was included as control. The samples were further incubated with FITC conjugated anti-CD63 and PE conjugated anti-CD203c monoclonal antibodies (clones CLBGran/12 and 97A6, respectively, Immunotech, Marseille, France) and analyzed by flow cytometry using a BD FACSCanto II (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo (Treestar, Ashland, OR). Basophils were identified by gating for CD203c positive cells and the magnitude of allergen-activation was calculated and expressed as the percentage of CD63 positive cells among the gated basophils.

Measurement of IgE cross-reactivity between Fel d 7 and Can f 1

The potential cross-reactivity between IgE to Fel d 7 and Can f 1 was investigated by inhibition ELISA. The sera were diluted to 1 kU_A/l and incubated at 1:1 v/v with 10-fold dilutions (range 10^{1}–10^{-7} μg/ml) of rFel d 7, rCan f 1 or PBS for 2 h at room temperature (5). Inhibition of IgE binding was analyzed by ELISA and calculated as $[(OD_{no\ inhibitor} - OD_{inhibitor})/OD_{no\ inhibitor}] \times 100$. Additionally, inhibition ELISA was performed using sera from two patients with IgE reactivity to rCan f 1 but not to rFel d 7.

Synthesis and characterization of Can f 1 derived peptides and immunization of rabbits

Six overlapping peptides spanning the Can f 1 sequence with a length between 30 and 36 amino acids were designed based on the prediction of surface exposure of amino acids as determined by the ProtScale bioinformatics tool from the ExPASY server (6) and were synthesized using an Applied Biosystems peptide synthesizer Model 433A (Foster City, CA). The peptides contained cysteins for coupling to keyhole limpet hemocyanin (KLH) either at N termini (Peptide 3, Peptide 4, Peptide 5 and Peptide 6) or at C termini (Peptide 1 and Peptide 2) (Table S1). One Peptide, P4, contains a highly hydrophobic region of Can f 1 and was not used in further studies due to non-solubility in water or biological buffers. All the other peptides were purified by preparative HPLC (Dionex, Thermofischer Scientific, Waltham, MA) and their identities were confirmed by mass spectrometry (Bruker, Bremen, Germany). Can f 1 peptides were coupled to KLH (Pierce, ThermoFisher Scientific, Waltham, MA) and purified using a conjugation kit according to manufacturer`s instructions. The concentration of KLH-conjugated Can f 1 peptides was measured using the Micro BCA Assay Kit (Pierce, Rockford, IL).

Peptide-specific IgG antibodies were obtained by immunizing rabbits three times (first booster injection after 4 weeks and a second booster injection after 7 weeks) with each of the KLH-conjugated peptides (200 µg/injection) and, for control purposes, with recombinant Can f 1 (200 µg/injection) using once Freund's complete and twice Freund's incomplete adjuvant (Charles River, Chatillon sur Chalaronnne, France). The "Directive 2010/63/EU of the European parliament and of the council (22 September 2010) on the protection of animals used for scientific purposes" were followed for the care and use of the animals. Five rabbit anti-Can f 1 peptide sera (diluted from 1:2000 to 1:50000) were used to evaluate cross-reactive binding sites between Can f 1 and Fel d 7. IgG binding was analyzed by ELISA using rFel d 7, rCan f 1 and rFel d 1 coated plates incubated with rabbit anti-Can f 1 peptide

antisera. Detection was performed with alkaline phosphatase conjugated goat-anti-rabbit IgG antibodies (1:1000, Jackson ImmunoResearch Laboratories, West Grove, PA). Detection was carried on like in IgE ELISA.

References

1. Smith W, O'Neil SE, Hales BJ, Chai TL, Hazell LA, Tanyaratsrisakul S, et al. Two newly identified cat allergens: the von Ebner gland protein Fel d 7 and the latherin-like protein Fel d 8. *Int Arch Allergy Immunol* 2011;**156**(2):159-170.
2. Madhurantakam C, Nilsson OB, Uchtenhagen H, Konradsen J, Saarne T, Hogbom 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**(1):68-83.
3. 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**(6):751-757.
4. Saarne T, Kaiser L, Gronlund H, Rasool O, Gafvelin G, van Hage-Hamsten M. Rational design of hypoallergens applied to the major cat allergen Fel d 1. *Clin Exp Allergy* 2005;**35**(5):657-663.
5. Madhurantakam C, Nilsson OB, Uchtenhagen H, Konradsen J, Saarne T, Hogbom E, et al. Crystal Structure of the Dog Lipocalin Allergen Can f 2: Implications for Cross-reactivity to the Cat Allergen Fel d 4. *Journal of Molecular Biology* 2010;**401**(1):68-83.
6. Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res* 2003;**31**(13):3784-3788.

Table S1. Characteristic of synthetic Can f 1-derived peptides.

No.	Position (AA)*	Sequence**	# AA	Molecular mass (Da)	pI
Peptide 1	1-35	*C*QDTPALGK**DT**VA**VSGKWYLKAM**TADQEVPE**KP**DS**V**	36	3879.37	4.53
Peptide 2	31-60	*C***KP**DS**VTPM**I**L**KAQ**KGGNL**E**A**KITML**TNGQ**	30	3187.78	9.11
Peptide 3	56-85	**TNGQC**QNITVV**L**H**KTSEP**G**KYT**A**Y**E**G**QRVV***C*	31	3424.85	7.74
Peptide 4	78-107	*C*A**Y**E**G**Q**RVV**F**I**Q**P**SP**V**R**DHYI**L**YCEGE**LH**G**R	31	3636.12	6.03
Peptide 5	101-132	*C***EGE**LHGR**Q**I**RMAKL**L**GRDPE**QSQ**EALE**D**F**W**F**	33	3919.36	4.59
Peptide 6	127-156	**E**D**F**W**EF**S**RAKG**L**NQEI**LELA**QS**E**TC**S**PG**GQ*C*	31	3473.79	4.14

*, Position of amino acid without signal peptide;
**, Bold and underlined sequences represent identical portions with Fel d 7;
C, Addition of cysteine.

Table S2. Serological characteristics of the 65 rFel d 7-positive patients.

Patient ID	Cat (kU_A/l)*	Dog (kU_A/l)*	rFel d 7 (kU_A/l)**	rCan f 1 (kU_A/l)**
1	2.2	19	2.3	7.9
2	3.4	14	7.2	13
3	44	2.9	4.6	2.5
4	14	16	37	33
5	4.5	21	18	20
6	24	12	11	8.3
7	19	27	7.4	15
8	110	29	11	18
9	2.6	0.26	2.5	0.64
10	190	58	14	29
11	5.2	0.62	2.2	0.76
12	60	7.2	28	9.9
13	50	7	3.5	1.5
14	17	12	9.1	11
15	24	10	2.8	4.0
16	6.6	2	2.6	2.4
17	9.7	3.4	2.6	1.9
18	2.5	7.4	2.0	1.9
19	160	59	1.9	26
20	44	36	1.6	31
21	14	26	1.4	17
22	16	11	1.3	2.2
23	11	1.5	1.3	0.87
24	6.1	0.87	1.2	0.38
25	5.1	0.19	1.1	0.27
26	14	1.1	1.1	1.4
27	16	0.86	1.0	<0.10
28	30	18	0.97	5.9
29	34	3.7	0.70	<0.10
30	19	8.9	0.62	1.2
31	3.2	0.12	0.53	0.43
32	9.4	16	0.44	29
33	7.2	0.97	0.43	0.17
34	14	36	0.35	0.89
35	3.8	0.69	0.16	<0.10
36	3	7.3	0.24	<0.10
37	2.7	25	0.16	19
38	9.6	10	0.93	4.2
39	29	11	28	26
40	165	11	27	4.8

41	82	13	28	26
42	6.0	41	2.8	1.4
43	189	17	0.26	<0.10
44	12	12	7.5	9.5
45	111	24	28	29
46	120	25	28	28
47	67	54	25	25
48	3.5	48	2.8	29
49	12	56	1.4	29
50	6.9	63	3.3	9.6
51	13	66	27	26
52	13	95	5.6	12
53	68	80	26	28
54	11	78	25	27
55	105	60	29	28
56	42	14	15	5.8
57	11	51	0.9	0.67
58	22	1.7	1.7	0.70
59	6.2	7.9	17	16
60	36	9.0	1.5	3.5
61	69	18	32	32
62	91	22	0.45	0.50
63	88	28	30.5	7.4
64	13	38	13	14
65	4.0	22	9.5	25

*****, Specific IgE levels in kU_A/l to cat and dog dander (ImmunoCAP; e1 and e5, respectively);

**, Allergen-specific IgE levels in kU_A/l (ELISA).

Table S3. Serological characteristics of patients in basophil activation test.

Patient	Cat (kU_A/l)*	Dog (kU_A/l)*	rFel d 7 (kU_A/l)**	rCan f 1 (kU_A/l)**
I	1.6	0.31	0.46	0.1
II	8.9	4.0	3.4	3.9
III	10	1.8	5.1	<0.10
IV	12	0.91	1.5	<0.10

*****, Specific IgE levels in kU_A/l to cat and dog dander (ImmunoCAP; e1 and e5, respectively);
******, Allergen-specific IgE levels in kU_A/l (ELISA).

Table S4. Percentages of secondary structure calculated in CDPro with CONTINILL algorithm using SP37 base.

Secondary structure	rFel d 7 (%)	rCan f 1 (%)	Δ sum of α and β (%)
α- helix	5.0	10.7	14.2
β- sheets	36.0	40.9	
β-turn	24.2	27.8	
unfolded	34.8	20.6	

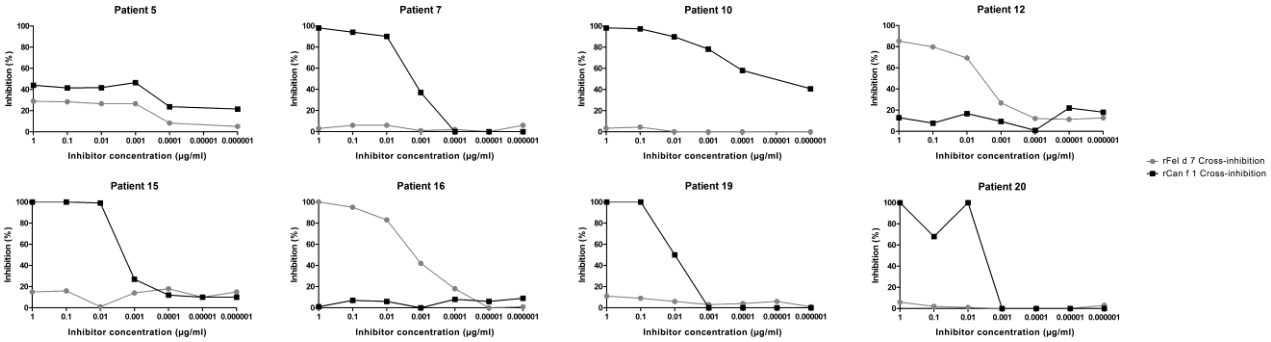


Figure S1 - IgE cross-reactivity between rFel d 7 and rCan f 1 in eight additional patients. Percentages of inhibition (y-axes) are plotted against different concentrations of inhibitor allergens (x-axes) in eight additional patients (5, 7, 10, 12, 15, 16, 19 and 20 in Table S2). IgE-binding to rCan f 1 in sera pre-incubated with rFel d 7–cross-inhibition (grey dots); IgE-binding to rFel d 7 in sera pre-incubated with rCan f 1–cross-inhibition (black squares).

A.

```
Fel d 7   1   MKALLLAVGLSLITVLQAQDPPASGEDTMAMSGKWYLKAMITDRETSWKKPELVTPMTLT   60
Can f 1   1   MKTLLLTIGFSLIAILQAQDTPALGKDTVAVSGKWYLKAMTADQEVP-EKPDSVTPMILK   59
P31025    1   MKPLLLAVSLGLIAALQAHHLLASDEEIQDVSGTWYLKAMTVDREFPEMNLESVTPMTLT   60
              ** ***::.:.**: ***:.  *  ::   :**.****** .*:*    : : **** *.
Fel d 7  61   VLEGGNLKAETTLLTNGQCKEVELILEKTSEPKKYTTYGGKRVVYIEPTEVKDHYIFYCE  120
Can f 1  60   AQKGGNLEAKITMLTNGQCQNITVVLHKTSEPGKYTAYEGQRVVFIQPSPVRDHYILYCE  119
P31025   61   TLEGGNLEAKVTMLISGRCQEVKAVLEKTDEPGKYTADGGKHVAYIIRSHVKDHYIFYCE  120
              . :****:*: *:* .*:*:::  :*.**.** ***:  *::*.:*  : *:****:***
Fel d 7 121   GEMQGEQARMAKLVGRDPESNEEALENFREFLRAKGFNQ-EIFSPKQSDTCPPGTDQEPEV 180
Can f 1 120   GELHGRQIRMAKLLGRDPEQSQEALEDFREFSRAKGLNQ-EILELAQSETCSPGGQ----- 174
P31025  121   GELHGKPVRGVKLVGRDPKNNLEALEDFEKAAGARGLSTESILIPRQSETCSPGSD----- 176
              **::*.  * .**:****:.. ****:*.:   *:*:.  .*:   **:** ** :
```

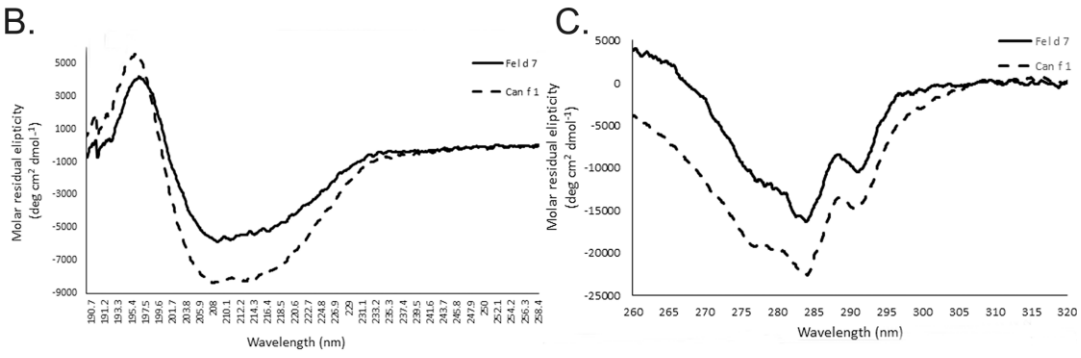
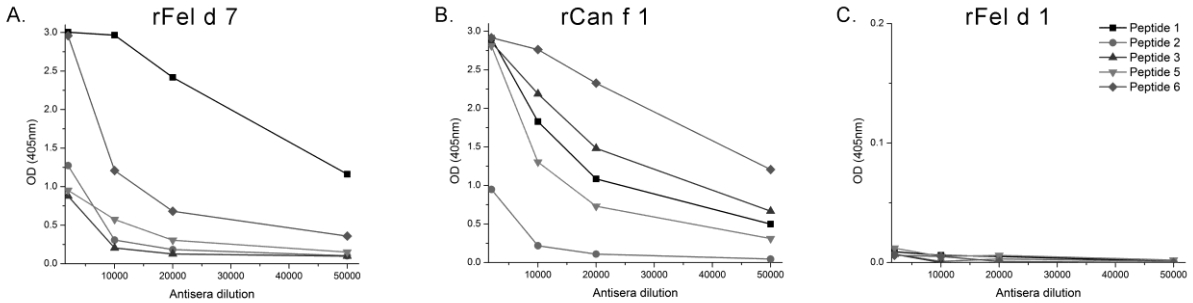


Figure S2 - Structural similarities between Fel d 7 and Can f 1. (A) Sequence alignment of Fel d 7, Can f 1 and human tear lipocalin (hTL). Underlined sequences show the signal peptide and symbols underneath the alignment indicate: positions which have a single, fully conserved residue (*), conservation between groups of strongly similar properties (:) and weakly similar properties (.). Percentages of sequence identity between: Fel d 7 vs Can f 1 63%, Fel d 7 and hTL 55%, Can f 1 and hTL 60%.; (B) Far UV CD spectra of rFel d 7 (solid line) and rCan f 1 (dashed line); (C) Near UV CD spectra of rFel d 7 (solid line) and rCan f 1 (dashed line).



198

199 **Figure S3** - Can f 1 peptide-specific IgG binding. (A) to rFel d 7; (B) to rCan f 1; (C) to the
200 unrelated allergen rFel d 1.Optical density values corresponding to allergen-specific IgG
201 levels (y-axis) of different dilutions of peptide-specific antisera (x-axis).