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Early View

Original article

Nasal upregulation of *CST1* in dog sensitised children with severe allergic airway disease

Ulrika Käck, Elisabet Einarsdottir, Marianne van Hage, Anna Asarnoj, Anna James, Anna Nopp, Kaarel Krjutskov, Shintaro Katayama, Juha Kere, Gunnar Lilja, Cilla Söderhäll, Jon R. Konradsen

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TITLE PAGE

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Summary message:

Nasal over-expression of CST1 reveals more severe allergic airway disease in children sensitized to dog dander, and is associated with lower FEV1%, bronchial hyper-reactivity, pronounced eosinophilia and higher basophil allergen threshold sensitivity.

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Abstract

Background: The clinical presentation of children sensitized to dog dander varies from asymptomatic to severe allergic airway disease, but the genetic mechanisms underlying these differences are not clear.

Objective: To investigate nasal transcriptomic profiles associated with dog dander sensitization in school children and to reveal clinical symptoms related with these profiles.

Methods: RNA was extracted from nasal epithelial cell brushings of children sensitized to dog dander and healthy controls. Blood sample analyses included IgE against dog dander, dog allergen molecules, other airborne and food allergens, basophil activation and white blood cell counts. Clinical history of asthma and rhinitis was recorded, and lung function was assessed (spirometry, methacholine provocation and FeNO).

Results: The most over-expressed gene in children sensitized to dog dander compared to healthy controls was *CST1*, coding for Cystatin 1. A cluster of these children with enhanced *CST1* expression showed lower FEV1, increased bronchial hyper-reactivity, pronounced eosinophilia and higher basophil allergen threshold sensitivity compared with other children sensitized to dog dander. Multi-sensitization to lipocalins was also more common in this group.

Conclusions: Over-expression of *CST1* is associated with more severe allergic airway disease in children sensitized to dog dander. *CST1* is thus a possible biomarker of the severity of allergic airway disease and a possible therapeutic target for the future treatment of airborne allergy.

Introduction

Dog allergy, mainly characterized by asthma and rhino-conjunctivitis, is affecting up to 10% of adolescents in the Western world. A prevalence of IgE reactivity to dog dander of up to around 20% is reported among teenagers in Nordic countries and the clinical presentation varies from asymptomatic to severe allergic airway disease [1-3]. There is still a gap in the understanding of factors determining why some individuals develop allergic disease and others only asymptomatic sensitization.

The first physical barrier for entry of allergens is the epithelial barrier, predominantly formed by tight junctions at the most apical part of neighboring epithelial cells [4, 5]. Impaired epithelial structure and function have been recognized as significantly contributing factors in the pathogenesis of asthma [6, 7], and variations in expression and DNA methylation of a number of genes in the airway epithelium have previously been associated with asthma and rhinitis [8-10].

Sampling from the nasal epithelium is an attractive alternative to investigate mechanisms of airborne allergic disease including the underlying transcriptomic profiles, as nasal epithelium gene expression reflects gene expression in the lower airways [11].

Molecular allergy diagnostics provides new opportunities to explore associations between sensitization to furry animals and clinical disease [12, 13]. Previously we provided a detailed characterization of sensitization profiles in a cohort of 60 dog dander-sensitized children, revealing multi-sensitization to dog allergen molecules and sensitization to lipocalins as important risk factors for dog allergy [3].

In this study, we further investigated the same cohort regarding associations between nasal epithelial gene expression, sensitization and clinical manifestations of allergic disease in detail. Our aim was to identify differences in gene expression between children sensitized to

dog dander and healthy controls, as well as to reveal transcriptomic profiles associated with more severe allergic airway disease.

Material and Methods

Patients

Fifty-eight dog dander-sensitized patients aged 10-18 years were recruited from pediatric outpatient clinics. Inclusion criteria consisted of confirmed sensitization to dog dander through a positive skin prick test (wheal size ≥3 mm) and/or serum IgE to dog dander ≥0.10 kU_A/l [3]. Patients were included regardless of history of clinical symptoms upon dog exposure. In addition, 20 age-matched healthy controls, without allergic symptoms of rhinitis or asthma were included through advertising at Sachs' children hospital, Stockholm. The study protocol was approved by the Regional Ethics Committee of Karolinska Institutet, Stockholm (Dnr 2014/1453-31/4). Written informed consent was obtained from parents and/or legal guardians.

Interviews and standardized questionnaires

All children and their guardians were interviewed using a standardized questionnaire which included questions regarding demographic data, such as family history of allergy and asthma, exposure to dogs and other furry animals, history of asthma, rhinitis and other allergic manifestations, symptom triggers, medication and healthcare use [14]. Asthma control was assessed using the Asthma control test (ACT) [15].

Blood sample analyses

Samples of venous blood were collected and white blood cell counts were measured. IgE against airborne allergens (dog-, cat- and horse dander, timothy, birch, mugwort, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and *Cladosporium herbarum*)

and the food mix fx5 (egg white, peanut, cow's milk, wheat, soy bean and codfish) were analyzed. Sera that scored positive (IgE ≥0.10 kU_A/l) for furry animal extracts were further analyzed for IgE against allergen molecules from dog (Can f 1- Can f 6), cat (Fel d 1, Fel d 2, Fel d 4) and horse (Equ c 1). Furthermore, sera showing an IgE ≥0.35 kU_A/l for fx5 were analyzed for the single allergens included in the mix. All IgE determinations were performed using the ImmunoCAP System (Thermo Fisher Scientific, Uppsala, Sweden) according to the manufacturer's instructions. The results are presented as kU_A/l and the cut-off level for single allergens was ≥0.10 kU_A/l.

The basophil activation test towards dog dander was performed and the basophil allergen threshold sensitivity (CD-sens) was calculated as previously described [16] [17].

Pulmonary function and bronchial hyper-reactivity

Spirometry and reversibility testing was performed using a Vitalograph™2120 (Vitalograph, Ennis, Ireland), in accordance with recommendations from the European Respiratory Society using the reference values reported by Polgar [18]. The fraction of exhaled nitric oxide (FeNO) was measured prior to spirometry (Niox Vero). Bronchial hyper-reactivity was assessed by methacholine challenge utilizing a Spira nebulizer (Spira Respiratory Care Center, Hämeenlinna, Finland) and PD20 (dose causing a 20% reduction in FEV1) was calculated [19].

Nasal provocation testing

Nasal provocation testing (NPT) was performed as previously described with a commercially available dog dander extract, Aquagen 100 000 SQ-U/ml (ALK-Abello, Copenhagen, Denmark) according to a modified Lebel protocol [3, 20]. One spray-dose, 0.1 ml, of diluted dog dander extract (10,000 SQ-U) was deposited in each nostril. Symptoms were recorded at

5, 15 and 30 min after administration and the sum of the score from the three occasions was calculated.

Nasal epithelial brushing

Nasal epithelial brushing was performed in cases and controls. Among cases, nasal provocation tests and nasal epithelial brushings were performed at different occasions, at least five days apart. Nasal epithelial cells were collected using a cervical cytology brush (Bastos Viegas, Penafiel, Portugal) from behind the inferior turbinate. Cells were immediately stored in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA), initially at 4°C overnight, followed by long term storage at -80°C until RNA extraction.

RNA extraction

Total RNA was extracted from nasal epithelial brushings using Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA quality and quantity were assessed using NanoDrop 8000, Qubit Fluorometric Quantitation (Thermo Fisher Scientific) and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), and an RNA integrity number ≥ 8 was used as cut-off for inclusion.

Transcriptome library preparation and sequencing

In total, 74 samples were included (54 cases and 20 healthy controls) and subdivided into 2 libraries. A modified version of the Single-cell Tagged Reverse Transcription (STRT) method [21], described in detail in [22] was used to prepare two 48-plex Illumina-compatible sequencing libraries from 20 ng of each epithelial RNA. The libraries were sequenced on four Illumina HiSeq2000 (Illumina, San Diego, CA, USA) lanes each, using the Illumina TruSeq

v3 60-bp single-read protocol. Sequencing was performed at the Bioinformatics and Expression Analysis core facility at Karolinska Institutet, Sweden.

Sequence data was converted to fastq files using Casava 1.8.2 (Illumina), and quality control performed using the STRTprep pipeline available at https://github.com/shka/STRTprep [22].

Statistical analyses

Differential expression and the statistical significance were tested by SAMstrt [21]. When comparing sample groups $q < 0.05$ was considered as significantly variable expression and genes with a q-value <0.05 and FC >1.2 or FC <0.5 were considered to be significantly differentially expressed. ClustVis (available at https://biit.cs.ut.ee/clustvis/) was used to generate heatmaps and principal component analysis (PCA) plots of the differentially expressed genes.

Analysis of clinical data was performed with Stata statistical software (release 14.1, Stata Corp, Texas, USA). Categorical data were presented as percentages and two-group comparisons performed using the Chi-Squared test, or Fisher's exact test when appropriate. T-test was used for group comparisons of log-transformed IgE values. The Mann Whitney test was used for other continuous variables. Correlations between *CST1*-levels, blood- and lung function parameters were investigated using Spearman rank order correlation. A p-value<0.05 was considered significant.

Results

Clinical characteristics

We investigated transcriptomic profiles in nasal epithelial brushings from a cohort of school-aged children. Participants sensitized to dog dander, including children with and without symptoms upon dog exposure, were compared to healthy non-sensitized controls. Figure 1 shows the flow chart leading to the final inclusion of 49 dog dander-sensitized cases and 17 healthy controls (Fig 1, Table E1). The children sensitized to dog dander had a lower FEV1 (102% vs. 110%, p=0.02), a higher median FeNO (32 vs. 15 ppb, p<0.001), a lower median score on the Asthma control test (22 vs. 25 points, p<0.001) and a higher blood eosinophil count (0.3 $x10^9$/l vs. 0.2 $x10^9$/l, p=0.001) compared to non-sensitized healthy controls. Interestingly, dog ownership was more common among the dog dander sensitized children than among the non-sensitized controls (Table 1).

Transcriptomic analysis

We found that 321 genes were significantly differently expressed among cases sensitized to dog dander compared to non-sensitized controls; 108 genes were upregulated in the cases while 213 genes were downregulated (q-value <0.05 and FC >1.2 or FC <0.5, Table E2). The most up-regulated gene among the cases was *CST1*, coding for Cystatin 1, with a median fold change 21 times higher than in controls (Table 2). The second most upregulated gene among the cases was *CCL26*, with a median fold change 4.5 times higher than in controls (Figure 2). The most downregulated gene in the cases was *CXCL-13*, coding for the C-X-C motif chemokine ligand 13 (FC 0.17 cases vs controls, Table 2).

Upregulation of CST1 and CCL26 in a cluster of cases

Unsupervised clustering of the samples according to the expression of the 10 most upregulated and 10 most downregulated genes identified a distinct cluster of ten individuals among the 49 dog dander-sensitized cases expressing higher levels of *CST1* and *CCL26* (Figure 3, Figure E1). The median fold change for *CST1* in this sub-group was > 500 times higher compared with the healthy controls and 47 times higher compared with other cases. Median fold change for *CCL26* was > 40 times higher than among controls (Figure 2). Hereafter, this sub-group of 10 children is referred to as "*CST1*-high".

Clinical characteristics of CST1-high children

CST1-high children showed a lower FEV1 (% predicted) compared to other children sensitized to dog (94 % vs 105 %, p=0.01), more pronounced bronchial airway responsiveness (PD20 methacholine 0.65 vs 2.34, p=0.04) and a tendency towards higher reversibility and higher FeNO levels (Figure 4A). As shown in Figure 4B they also had a higher median *in vitro* basophil allergen threshold sensitivity (CD-sens) towards dog dander than the other children sensitized to dog (1.8 vs 0.20, p=0.01). Further, blood eosinophil counts were significantly higher among *CST1*-high children (0.65 $x10^9$/l vs 0.3 $x10^9$/l, p=0.02) (Figure 4B). The *CST1*-high group also displayed an earlier onset of rhino-conjunctivitis, but frequencies of reported rhino-conjunctivitis and asthma were similar between the two groups, as were the results of nasal provocation tests with dog dander extract. We did not see differences in cat or dog ownership between these two groups (Table E3).

Molecular allergy diagnostics in CST1-high children

The *CST1*-high children were in a greater extent multi-sensitized towards dog allergen molecules than the other children sensitized to dog (median 5 vs 2 allergen molecules, p=0.04). Furthermore, multi-sensitization to lipocalins from dog, cat and horse was more

common in this group (median 6 vs 3 lipocalins, p=0.03). Regarding single allergen molecules, the frequencies of children sensitized to the dog lipocalins Can f 2 and Can f 6 were higher in the *CST1*-high group than among the other children sensitized to dog (80% vs. 42% and 80% vs. 36%, respectively) (Figure 5, Table E4).

Despite the higher basophil allergen sensitivity among *CST1*-high children, IgE levels against dog dander did not differ significantly between the groups (46 *vs* 8.9 kU_A/l, p=0.12) (Table E5). Furthermore, no significant difference in IgE levels towards any furry animal allergen molecule was noted between *CST1*-high children compared to other children sensitized to dog, nor did sensitization rates for specific food or airborne allergens differ between the groups (Table E4, Table E5).

Correlations between CST1, inflammatory markers and lung function

Since the *CST1*-high cluster was not uniquely identified by higher *CST1* levels but according to the ten most up-and downregulated genes, we further analyzed correlations between expression levels of the most upregulated gene *CST1* and lung function parameters and blood cell count. We found a significant correlation between *CST1* levels and FeNO (r_s= 0.55, p<0.001) and a negative correlation between *CST1* levels and PD20 methacholine, (r_s= -0.56, p<0.001). We also found weaker, but significant correlations between *CST1* levels and impaired FEV1% (r_s= -0.37, p< 0.002), spirometry reversibility (r_s= 0.32, p=0.009) and blood eosinophil count (r_s= 0.47, p<0.001).

Discussion

To our knowledge this is the first study to investigate associations between nasal gene expression and multiple clinical characteristics of children sensitized to dog. We found several genes that were up-regulated in the nasal airways of children sensitized to dog compared to healthy controls, the highest of which was *CST1*, with more than a 20-fold up-regulation. Among the children sensitized to dog dander, there was a cluster of children demonstrating a marked nasal upregulation of *CST1* and *CCL26*, and these children had impaired lung function, pronounced eosinophilia, higher basophil allergen threshold sensitivity and more frequent multi-sensitization towards dog allergen molecules.

Our data underline the heterogeneity in clinical presentation among children sensitized to dog dander, from being asymptomatic to having persistent rhinitis and asthma. By analyzing differences in nasal transcriptomic profiles, we could identify a distinct cluster of children among those sensitized to dog dander with more severe allergic disease. These transcriptomic profiles can provide information on possible underlying mechanisms and may serve as useful biomarkers.

CST1 codes for the protein Cystatin 1 (also called Cystatin SN), belonging to a group of type 2 cysteine protease inhibitors. In humans CST1 is specifically highly expressed in salivary glands and there are hundreds of minor salivary glands in the nasal cavity. Cystatin 1 has recently been suggested to play a crucial role in the activation and recruitment of eosinophils in the nasal epithelium of patients with eosinophilic chronic rhino-sinusitis with nasal polyps [23], and has been proposed as a biomarker for seasonal allergic rhinitis as well as for eosinophilic chronic rhino-sinusitis with nasal polyposis [24, 25]. However, findings regarding *CST1* are contradictory as the corresponding protein, Cystatin 1 has also been shown to inhibit allergen protease activity, suppress allergic rhinitis symptoms and protect the nasal epithelial barrier [26, 27].

We mainly observed pronounced symptoms from the lower airways among *CST1*-high children, suggesting that *CST1*-upregulation also plays a role in allergic disease of the lower airways. These findings are supported by George *et al.* who found bronchial *CST1* up-regulation among adults with eosinophilic COPD and asthma [28]. Further, upregulated *CST1* transcripts in nasal and bronchial epithelia has been patented as one of several IL13-dependent biomarkers that can identify patients with a high risk of asthma exacerbation [29]. It is known that pulmonary airway gene expression is reflected by gene expression in the upper airways, and recently, it has also been shown that transcriptional variations associated with airway disease are present in both nasal and tracheal epithelium [30]. Gene expression is affected by epigenetic mechanisms, such as DNA methylation. Interestingly, DNA methylation differences associated to asthma, rhinitis and allergy have been shown in nasal epithelium from children, which may facilitate our understanding of gene-environment interactions [10].

The impact of exposure to dogs and other furry animals on allergic airway disease in sensitized individuals is well established [31]. Dog ownership was more common among the dog dander sensitized children than among the healthy controls, though we did not see differences in exposure between the *CST1*-high expressing group and other dog dander sensitized children, indicating that dog exposure per se cannot explain the increase in *CST1*-expression.

We found higher basophil threshold sensitivity (CD-sens) towards dog dander among *CST1*-high children, whereas IgE levels against dog dander did not differ significantly between *CST1*-high and other children sensitized to dog. CD-sens expresses the *in vitro* effect of the allergen on basophils and thus provide a response that corresponds to the individual's clinical reaction upon allergen exposure [32]. Our finding indicates that the *CST1*-high expressing children have a higher biological allergen sensitivity compared to *CST1*-low children. When

investigating IgE to allergen extracts there is a risk of cross reactivity of unknown clinical relevance, making the association between IgE levels and clinical presentation less reliable, which probably explain the lack of difference in IgE levels to dog dander between the two groups in our material. We have previously shown that children sensitized to dog, yet tolerant of dogs, had a lower CD-sens level than children with manifest dog allergy and that children with severe allergic asthma have increased basophil allergen sensitivity also compared to children with mild to moderate asthma [16, 33]. It has previously been shown that eosinophil recruitment in the nasal mucosa is enhanced by Cystatin 1 locally, but also blood eosinophil levels were higher among individuals with high nasal *CST1* expression suggesting systemic differences between individuals with high and low nasal *CST1*-expression [23].

Lipocalins represent the most important group of inhaled dog allergen molecules. We have previously shown that multi-sensitization to dog allergens and sensitization to lipocalins is associated with increased morbidity [3, 13]. The pan-European research network MEDALL (Mechanisms of the Development of Allergy) has introduced the concept that mono- and poly-sensitized individuals represent different phenotypes, where poly-sensitization is associated with multi-morbidity and more severe disease [34].

Despite the absence of significant difference in IgE-levels to dog dander *CST1*-high expressing children showed more frequently poly-sensitization to dog allergens and lipocalins, displayed more impaired lung function and higher inflammatory markers compared to those with lower *CST1* expression. Thus, increased nasal *CST1* expression could be one possible marker for this polysensitized phenotype.

Taken together, our study show that nasal *CST1* expression is associated with clinical and biochemical markers for asthma and airway allergy, suggesting that *CST1* has the potential of being a biomarker of the morbidity in individuals with allergic sensitization.

Nevertheless, as this is a small study on children sensitized to dog dander, our findings need to be replicated in larger cohorts across all ages, sensitized to a wider range of allergens to further elucidate the potential role of CST1 as a biomarker and therapeutic target in allergic patients.

Strengths and limitations: A major strength of this study is the use of nasal epithelial brushings as many genes show cell-type specific expression patterns. Nasal epithelium is the first physical barrier against airborne allergens, and nasal brushings are therefore highly relevant to study in sensitized children. Importantly, the gene expression profiles in nasal airways have shown high similarity with gene expression profiles in the bronchial and small airways [8], indicating that the results also are applicable for children with asthma. An important limitation is the size of the study population, with only ten individuals in the *CST1*-high group, making the analysis less robust so that minor differences between the groups might not be revealed. Although previous studies support our results, future studies evaluating the expression of these genes in children sensitized to dog, are required, specifically as we did not have the opportunity to replicate the results in a similar cohort. This is particularly important as differences in gene expression between the whole dog dander sensitized group and healthy controls were small.

Conclusion: *CST1* was the most over-expressed gene in children sensitized to dog dander compared to healthy controls. Among children expressing higher levels of *CST1* we found a lower FEV1, increased bronchial hyper-reactivity, pronounced eosinophilia and higher basophil allergen threshold sensitivity towards dog dander, providing further evidence that *CST1* may be an important mediator and biomarker of allergic disease. Thus, the gene expression profile of the airway mucosa of children sensitized to dog may contribute to our understanding of the pathogenesis of dog allergy, as well as provide biomarkers and targets for the development of new treatments.

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Author contribution

UK Patient inclusion, gathering and analysis of clinical data and manus drafting, EE Seq project management, bioinformatics, MvH IgE analyses and manuscript revision, AA Involved in analysis of clinical data, AJ Involved in study design, sample acquisition and RNA extraction, AN Supervising Basophil activation testing and CD-sens analysis, KK RNA-seq project laboratory setup and analysis, SK Bioinformatics and the resource management, GL Involved in study design and manuscript revision, JK Supervised gene expression analyses, CS Conceived and supervised the gene expression analyses, interpretation of the results, manuscript drafting, JRK Conceived study design, interpretation of the results.

All authors participated in revising the manuscript and approved the final version.

Conflict of Interest Statement

Dr. Käck reports lecture fees from Thermo Fisher, outside the submitted work. Dr. Katayama reports grants from Jane & Aatos Erkko Foundation, during the conduct of the study. Dr. van Hage reports personal fees from Thermo Fisher Scientific, personal fees from Hycor Biomedical LLC, CA, US., outside the submitted work. Dr. Konradsen has received material from Thermo Fisher Scientific to perform the IgE analysis in this project.

The rest of the authors declare that they have no relevant conflicts of interest.

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Tables:

Table 1. Demographic and clinical characteristics of dog dander sensitized children and healthy controls (IQR; Inter quartile range. SD; Standard deviation).

	Dog dander sensitized cases n=49	**Healthy controls n=17**	**p-value**
Female gender	35 % (n=17)	53 % (n=9)	0.19
Age mean years (range)	13.2 (10-17)	13.3 (10-17)	0.88
Exposure to dogs			
Dog at home, n (%)	12 (24 %)	0 (0%)	**0.03**
Dog in the catchment area, n (%)	39 (80 %)	11 (65 %)	0.32
Lung function	n=49	n=17	
FEV 1 %, (IQR)	102 (93-109)	110 (99-119)	**0.02**
Reversibility % median (IQR)	5.7 (1.8-11.5)	3.7 (1.6-5.9)	0.12
FeNO ppb median (IQR)	32 (20-59)	15 (11-18)	**<0.001**
Asthma control test median (IQR)	(n=48) 22 (19-24)	25 (25-27)	**<0.001**
White blood cell count	n=48	n=17	
Leukocytes, 10^9/l median (IQR)	5.9 (5.1-7.5)	6.8 (6.0-7.6)	0.28
Neutrophils, 10^9/l median (IQR)	2.9 (2.3-4.1)	3.2 (2.9-4.8)	0.13
Eosinophils, 10^9/l median 10^9/L(IQR)	0.3 (0.25-0.6)	0.2 (0.1-0.2)	**0.001**

Table 2 - The top 10 most significantly up- or downregulated genes and their fold change (FC) in dog sensitized children (cases) vs healthy controls.

Gene	**Gene name**	**Fold Change**
Upregulated in cases		
CST1	Cystatin-SN	21.33
CCL26	Chemokine (C-C motif) ligand 26	4.58
FOXQ1	Forkhead box Q1	4.51
ZG16	Zymogen granule protein 16	2.75
MTRNR2L6	MT-RNR2-like 6	2.02
ZNF398	Zinc finger protein 398	1.99
XKRX	XK related X-linked	1.94
IFT122	Intraflagellar transport 122	1.82
DEFB1	Defensin beta 1	1.70
KCNK7	Potassium two pore domain channel subfamily K member 7	1.68
Downregulated in cases		
CXCL13	C-X-C motif chemokine ligand 13	0.17
SIAH3	Siah E3 ubiquitin protein ligase family member 3	0.20
ATPAF1	ATP synthase mitochondrial F1 complex assembly factor 1	0.21
LRRC45	Leucine rich repeat containing 45	0.22
TMIGD2	Transmembrane and immunoglobulin domain containing 2	0.23
FAM64A	Family with sequence similarity 64, member A	0.23
AGAP3	ArfGAP with GTPase domain, ankyrin repeat and PH domain 3	0.24
KIAA0226L	Rubicon like autophagy enhancer (RUBCNL)	0.24
PZP	Alpha-2-macroglobulin like	0.24
ULBP3	UL16 binding protein 3	0.25

Figure captions:

Figure 1: Flow chart from patient inclusion to final study population. Total RNA was obtained from 54 children sensitized to dog dander recruited from outpatient pediatric clinics in the Stockholm area and 20 age matched healthy controls from the same area recruited through advertising. The sequencing yielded approx. 11-12M reads/sample (Table E1). Five case samples were removed as part of the QC analysis (three had too few spike-ins for high quality normalisation, two showed degraded RNA). Three of the healthy controls showed to be sensitized to dog dander (IgE>0.10 kU_A/l) and were therefore excluded from further analysis. For each of the technical duplicates passing QC, the sample with the highest number of raw reads was retained. This resulted in a final set of 49 independent cases and 17 healthy controls.

Figure 2: Expression of *CST1* (coding for Cystatin-SN) and *CCL26* (coding for the C-C motif chemokine ligand 26) among healthy control children (n=17) and dog dander sensitized children. Dog dander sensitized children are separated into *CST1* high "cluster cases" (n=10) and low "non-cluster cases" (n=39) sub-groups.

Figure 3: Heatmap based on the top 10 most up- or downregulated genes in cases versus controls. Cases are marked in red, controls in blue (top bar), hierarchical clustering shows the unsupervised clustering of samples. Red indicates relative higher expression, blue lower expression.

Figure 4: **A**. Lung function parameters in *CST1*-high children compared to other dog dander-sensitized children. FEV1; FEV1 (% predicted) before reversibility test with short acting β2-agonist.

Reversibility; % change in FEV1 after administration of short acting β2-agonist.
BHR; Bronchial hyper-reactivity, dose of Methacholine leading to a 20% drop in FEV1 (Methacholine PD20). Two children were excluded due to having FEV1 ≤75% of expected at baseline, two children were excluded due to technical issues and six children did not reach a drop in FEV1>20% during the bronchial provocation.
B. Blood cell counts (levels of neutrophils and eosinophils) and dog dander CD-sens levels among *CST1*-high children (n=10) compared to other children sensitized to dog dander (n=38).

Figure 5: Proportions of children (%) sensitized to dog allergen molecules among *CST1*-high (red, n=10) and other children sensitized to dog dander (blue, n=39).

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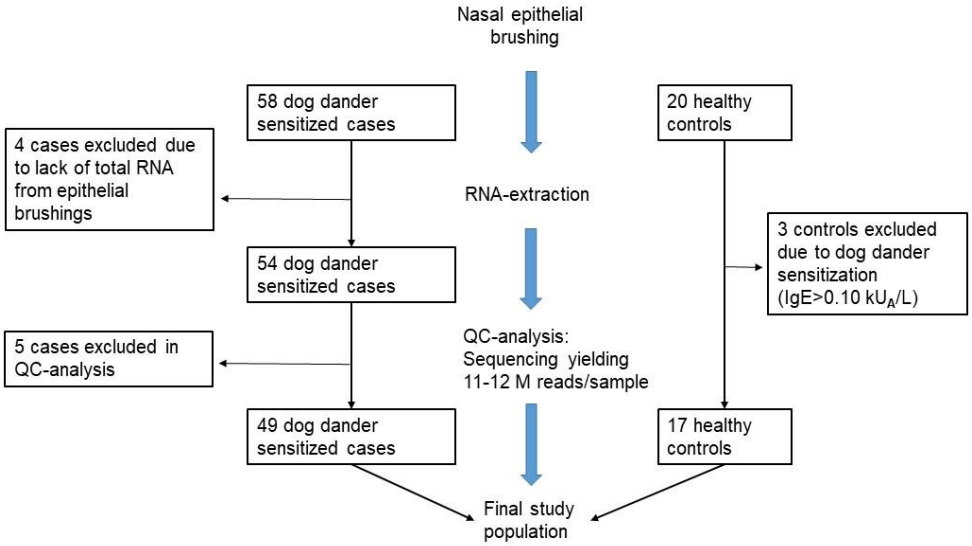
Figure E1: Principal component (PC) plot showing the first two PC components (PC1 and PC2), based on the ten most highly up and down-regulated genes. Cases are shown in red dots, controls in blue.

Table E1: Sequencing QC summary, including sequencing yield and mapping rates.

Table E2: Full list of significantly up- and downregulated genes based on normalized expression values for all samples. q-value <0.05 and FC > 1.2 (marked in red) or FC < 0.5 (marked in blue) were considered to be significantly differentially expressed.


Nasal epithelial brushing
58 dog dander sensitized cases
20 healthy controls
4 cases excluded due to lack of total RNA from epithelial brushings
RNA-extraction
3 controls excluded due to dog dander sensitization (IgE>0.10 kU$_A$/L)
54 dog dander sensitized cases
5 cases excluded in QC-analysis
QC-analysis: Sequencing yielding 11-12 M reads/sample
49 dog dander sensitized cases
17 healthy controls
Final study population

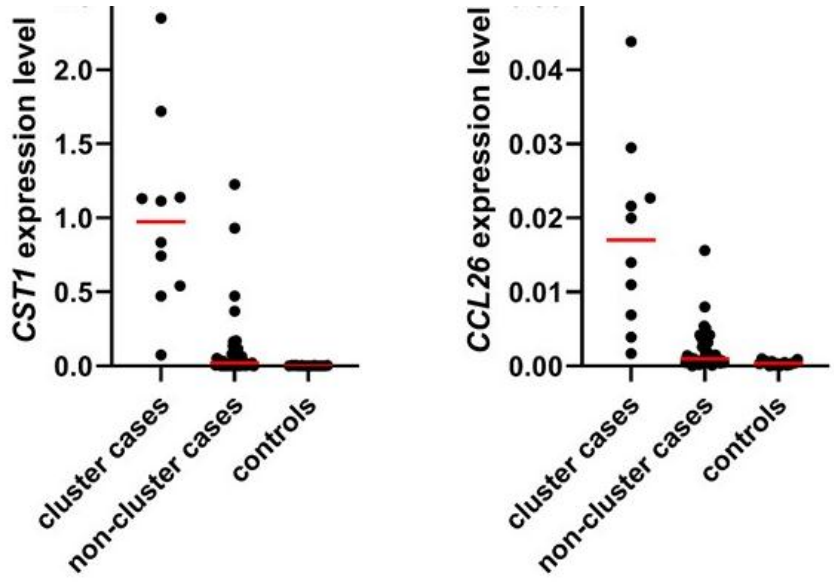
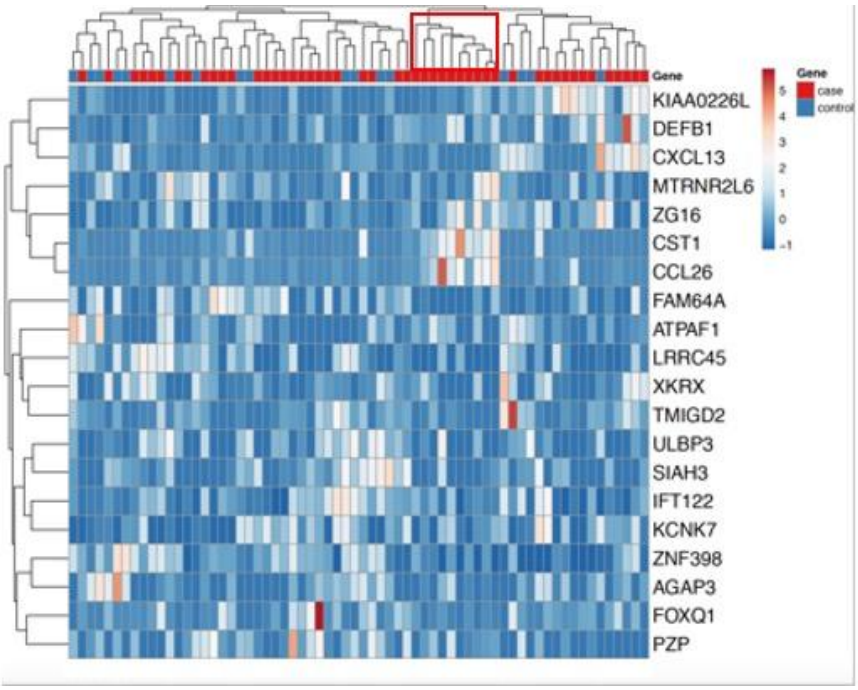
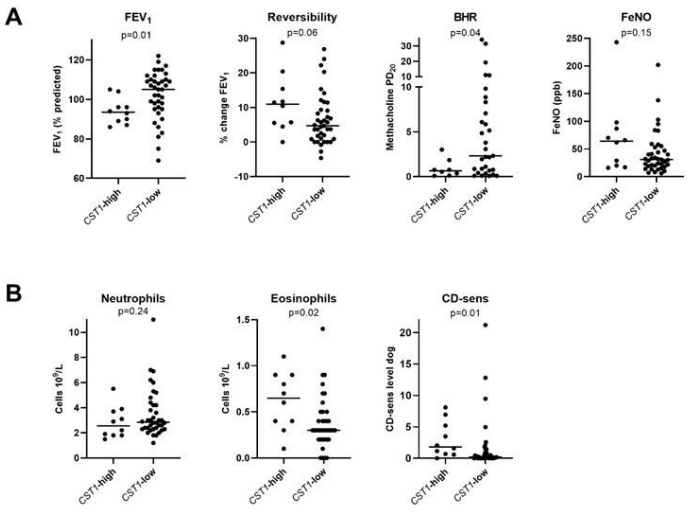

CST1 expression level
2.0
1.5
1.0
0.5
0.0
cluster cases
non-cluster cases
controls
CCL26 expression level
0.04
0.03
0.02
0.01
0.00
cluster cases
non-cluster cases
controls

Figure 2

Figure 3


Gene
KIAA0226L
DEFB1
CXCL13
MTRNR2L6
ZG16
CST1
CCL26
FAM64A
ATPAF1
LRRC45
XKRX
TMIGD2
ULBP3
SIAH3
IFT122
KCNK7
ZNF398
AGAP3
FOXQ1
PZP
Gene
case
control
5
4
3
2
1
0
-1


A
FEV1
p=0.01
FEV1 (% predicted)
120
100
80
60
CST1-high
CST1-low
Reversibility
p=0.06
% change FEV1
30
20
10
0
-10
CST1-high
CST1-low
BHR
p=0.04
Methacholine PD20
30
20
10
10
5
0
CST1-high
CST1-low
FeNO
p=0.15
FeNO (ppb)
250
200
150
100
50
0
CST1-high
CST1-low
B
Neutrophils
p=0.24
Cells 10^9/L
10
8
6
4
2
0
CST1-high
CST1-low
Eosinophils
p=0.02
Cells 10^9/L
1.5
1.0
0.5
0.0
CST1-high
CST1-low
CD-sens
p=0.01
CD-sens level dog
20
15
10
5
0
CST1-high
CST1-low

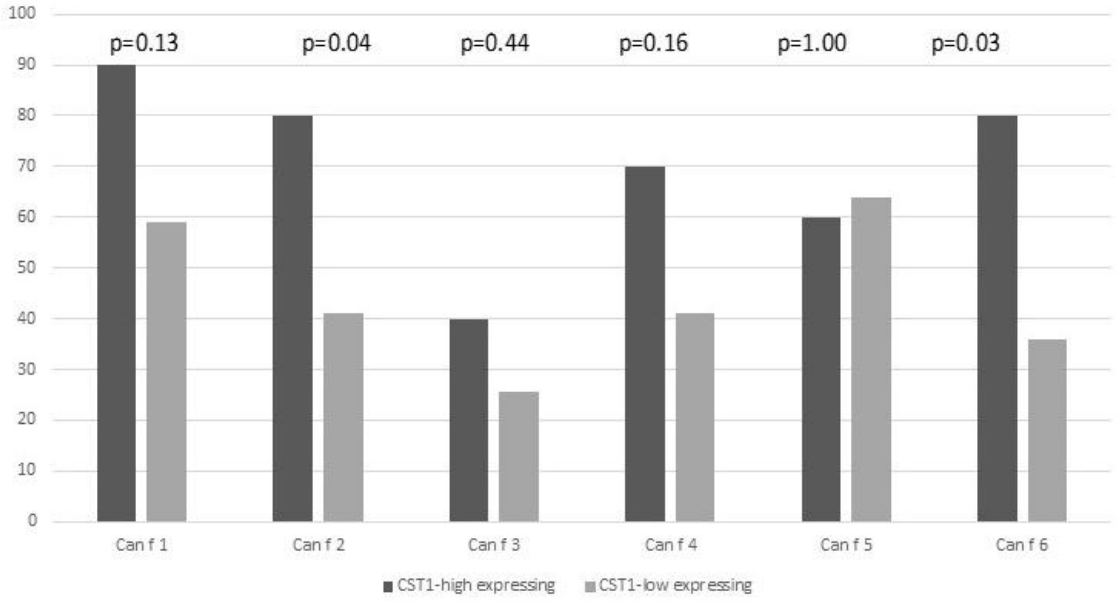
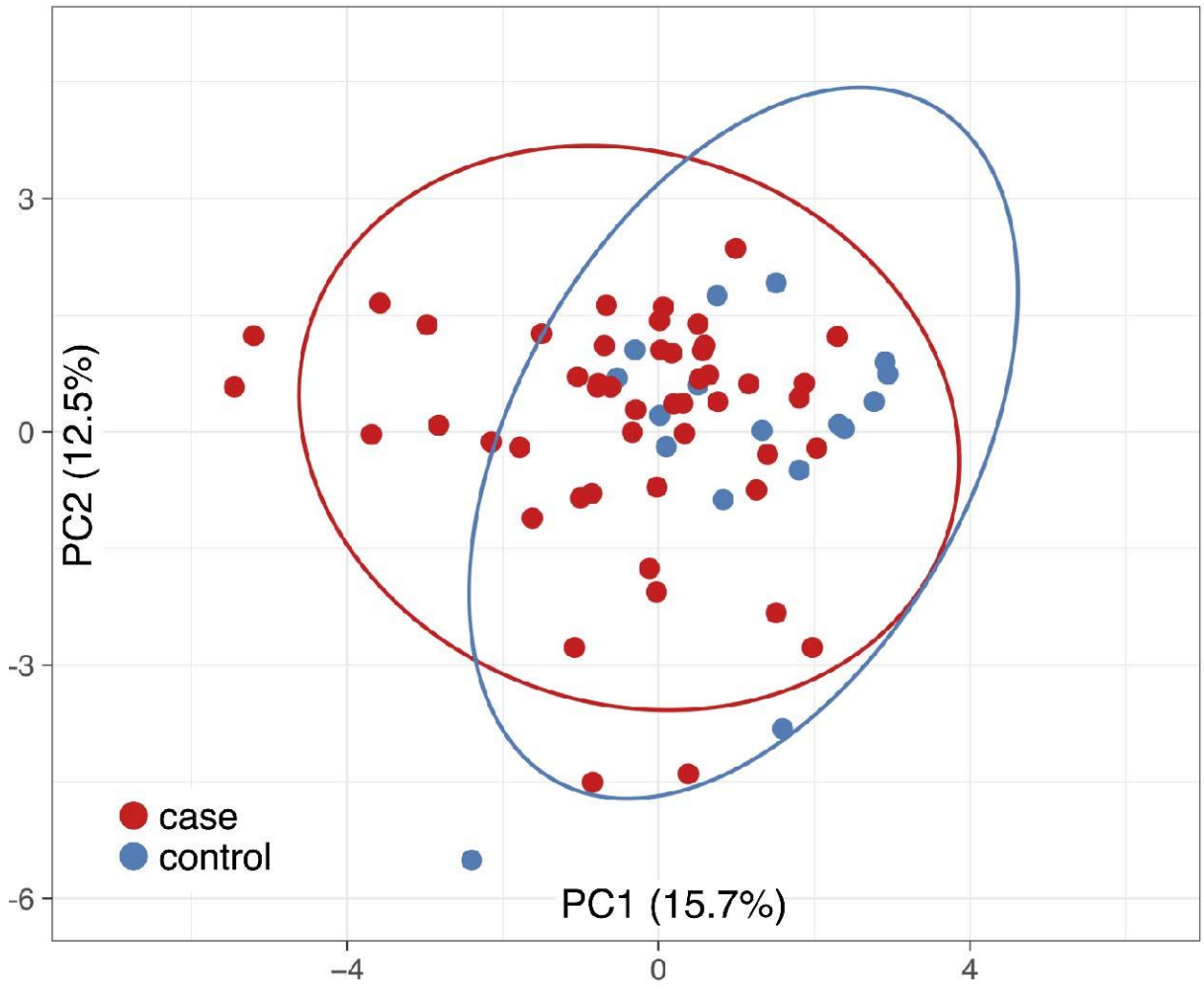

100
90
80
70
60
50
40
30
20
10
0
p=0.13
p=0.04
p=0.44
p=0.16
p=1.00
p=0.03
Can f 1
Can f 2
Can f 3
Can f 4
Can f 5
Can f 6
CST1-high expressing
CST1-low expressing

Figure 5.


3
0
-3
-6
PC2 (12.5%)
case
control
−4
0
4
PC1 (15.7%)

Online repository

Nasal gene expression profiling in children sensitized to dog dander identifies upregulation of *CST1* in more severe allergic disease

Figure E1: Principal component (PC) plot showing the first two PC components (PC1 and PC2), based on the ten most highly up and down-regulated genes. Cases are shown in red dots, controls in blue.

Table E1: Sequencing QC summary, including sequencing yield and mapping rates.

Table E2: Full list of significantly up- and downregulated genes based on normalized expression values for all samples. q-value <0.05 and FC > 1.2 (marked in red) or FC < 0.5 (marked in blue) were considered to be significantly differentially expressed.

Table E3: History of allergy and asthma among children with high expression of *CST1* and other dog dander sensitized children.

	CST1-high expressing (n=10)	Other dog dander sensitized (n=39)	p-value
HEREDITY n (%)			
Dog allergy, any parent	1/10 (5 %)	14/39 (36 %)	0.15
Asthma, any parent	2/10 (20 %)	18/39 (46 %)	0.17
Rhino conjunctivitis, any parent	7/10 (70 %)	27/39 (69 %)	1.00
EXPOSURE TO FURRY ANIMALS			
Dog at home	2	10	1.00
Dog in the catchment arera	6	33	0.18
Cat at home	0	2	1.00
Cat in the catchment arera	4	25	0.28
RHINITIS n (%)			
Reported rhinitis	9 (90 %)	39 (100 %)	0.20
Reported rhinitis at dog exposure	7 (70 %)	27 (69 %)	1.00
Nasal composite score Median points (IQR)	19 (14-22)	14 (8-21)	0.31
Age at onset (any rhinitis) Years, median (IQR)	2 (2-2)	4 (2-7)	**0.04**
ASTHMA n (%)			
Reported asthma	9 (90 %)	33 (84 %)	1.00
Reported asthma at dog exposure	5 (50 %)	20 (51 %)	0.94
Inhaled steroids regularly	6 (60 %)	23 (58 %)	1.00
Asthma control test Median (IQR)	22.5 (20-25)	22 (19-24)	0.52
Age at onset, years Median (IQR)	2.5 (1.5-9)	5 (2-8)	0.74
ECZEMA n (%)			
Ever eczema	5 (50 %)	25 (64 %)	0.48
Current eczema	4 (40 %)	17 (44 %)	1.0
Age at onset (months, IQR))	2 (1-3)	6 (3-12)	0.064

Table E4: Multi-sensitization to furry animal allergens and sensitization rates to cat and horse allergens, other airborne allergens and food among *CST1*-high expression cluster and the other dog dander sensitized cases.

Sensitization >0.1 kU/l	*CST1*-high expressing n=10	Other dog dander sensitized n=39	p-value
Multi-sensitization, Median (IQR)			
No. positive dog allergen molecules	5 (3-6)	2 (1-4)	**0.04**
No. positive lipocalins	6 (3-6)	3 (1-5)	**0.03**
Sensitization rates, n (%)			
Cat dander	9 (90)	38 (97)	0.37
Fel d 1	8 (80)	32 (82)	1.00
Fel d 2	5 (50)	9 (23)	0.12
Fel d 4	7 (70)	24 (61)	0.73
Horse dander	8 (80)	31 (79)	1.00
Equ c 1	7 (70)	23 (59)	0.72
D. pteronyssinus	8 (80)	30 (77)	1.00
D. farinae	7 (70)	28 (72)	1.00
Thimothy	8 (80)	36 (92)	0.27
Cladosporium	5 (50)	18 (46)	1.00
Birch	7 (70)	31 (79)	0.67
Mugwort	3 (30)	15 (38)	0.73
Egg white	8 (80)	18 (46)	0.08
Peanut	7 (70)	20 (51)	0.48
Cow’s milk	8 (80)	20 (51)	0.16
Wheat	6 (60)	23 (39)	1.00
Soybean	7 (70)	18 (46)	0.29
Codfish	3 (30)	7 (18)	0.41
Fx5	9 (90)	36 (92)	1.00

Table E 5: IgE to other furry animals, airborne allergens and food allergens: Levels among sensitized. Levels above 100 kU_A/L were set at 100 kU_A/L for specific IgE and IgE to allergen molecules.

IgE-titer kU_A/l (IQR)	*CST1*-high expressing (n=10)	Other dog dander sensitized (n=39)	p-value (t-test on log values)
Total IgE	638 (250- 1169)	414 (198- 781)	0.66
Dog allergens			
Dog dander	46 (3.7-92)	8.9 (2.7-37)	0.12
Can f 1	14 (2.3-31)	4.7 (2.1-31)	0.57
Can f 2	20 (3.45-45.5)	2.9 (0.34-16)	0.08
Can f 3	1.8 (1.165-12.9)	0.95 (0.17-6.1)	0.62
Can f 4	3.29 (0.31-8.31)	0.99 (0.47-2.46)	0.15
Can f 5	4.8 (0.47-36)	2.3 (0.71-8.2)	0.58
Can f 6	0.76 (0.27-4.16)	0.72 (0.37-1.0)	0.54
Cat allergens			
Cat dander	9.53 (4.96-87.4)	7.32 (2.50-19.1)	0.11
Fel d 1	38.4 (5.29-83.6)	6.55 (2.32-24.5)	0.13
Fel d 2	0.33 (0.25-0.72)	0.65 (0.25-9.97)	0.40
Fel d 4	1.63 (0.50- 11.5)	3.39 (0.49- 13.6)	0.67
Horse allergens			
Horse dander	16.1 (7.05-43.5)	4.69 (0.58- 19.0)	0.07
Equ c 1	10.4 (1.36- 32.0)	5.85 (2.36- 15.8)	0.78
Other airborne allergens			
D. peronyssius	0.63 (0.25- 44.6)	0.52 (0.19- 3.60)	0.61
D. farinae	0.79 (0.33- 100)	0.76 (0.22- 2.73)	0.53
Thimothy	16.5 (3.88- 81.2)	5.42 (0.93- 53.0)	0.40
Cladosporium	3.15 (2.12- 4.22)	0.65 (0.15-3.10)	0.15
Birch	29.5 (0.15-100)	7.39 (1.06-43.2)	0.82
Mugwort	0.62 (0.29- 4.09)	0.75 (0.31-2.67)	0.91
Food allergens			
Egg white	0.82 (0.37- 6.68)	0.82 (0.34-1.88)	0.65
Peanut	10.8 (1.5-100)	1.46 (0.72-8.00)	0.09
Cow's milk	0.45 (0.22- 2.00)	0.32 (0.15-1.03)	0.45
Wheat	2.09 (0.78- 5.15)	0.57 (0.23- 2.01)	0.07
Soybean	2.88 (0.57-5.32)	0.53 (0.29- 3.07)	0.16
Codfish	0.22 (0.21- 0.30)	0.15 (0.15- 0.19)	**0.02**
Fx5	8.34 (1.04-55.6)	0.63 (0.25-5.28)	**0.03**