Epithelium-derived Cystatin-SN enhances eosinophil activation and infiltration via interleukin-5 in chronic rhinosinusitis with nasal polyps

Bing Yan, Ph.D., Hongfei Lou, M.D., Ph.D., Yang Wang, B.S., Ying Li, B.S., Yifan Meng, M.D., Ph.D., Sihan Qi, M.D, Ming Wang, Ph.D., Lei Xiao, M.D., Ph.D., Chengshuo Wang, M.D., Ph.D., Luo Zhang, M.D., Ph.D.

PII: S0091-6749(19)30482-8
DOI: https://doi.org/10.1016/j.jaci.2019.03.026
Reference: YMAI 13960

To appear in: Journal of Allergy and Clinical Immunology

Received Date: 30 May 2018
Revised Date: 14 March 2019
Accepted Date: 22 March 2019


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Title: Epithelium-derived Cystatin-SN enhances eosinophil activation and infiltration via interleukin-5 in chronic rhinosinusitis with nasal polyps

Authors: Bing Yan, Ph.D.\textsuperscript{1,2}, Hongfei Lou, M.D., Ph.D.\textsuperscript{1}, Yang Wang, B.S.\textsuperscript{1,2}, Ying Li, B.S.\textsuperscript{1,2}, Yifan Meng, M.D., Ph.D.\textsuperscript{1}, Sihan Qi, M.D., \textsuperscript{1,2}, Ming Wang, Ph.D.\textsuperscript{1,2}, Lei Xiao, M.D., Ph.D.\textsuperscript{3}\#, Chengshuo Wang, M.D., Ph.D.\textsuperscript{1,2,\ast}, Luo Zhang, M.D., Ph.D.\textsuperscript{1,2,4}\ast

#: Lei Xiao is now working at Lung Cell and Vascular Biology Program, National Heart, Lung and Blood Institute, National Institutes of Health (NIH)

*: These authors contributed equally to the study.

Affiliations: 1 Department of Otolaryngology, Head and Neck Surgery, Beijing TongRen Hospital, Capital Medical University, Beijing 100730, China.

2 Beijing Key Laboratory of Nasal Diseases, Beijing Institute of Otolaryngology, Beijing 100005, China.

3 Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA.

4 Department of Allergy, Beijing TongRen Hospital, Capital Medical University, Beijing 100730, China.

Corresponding authors:

Luo Zhang, M.D., Ph.D.,
Beijing TongRen Hospital, Capital Medical University
No. 1, DongJiaoMinXiang, DongCheng District, Beijing, China
E-mail: dr.luozhang@139.com
Mobile phone: +86 13910830399
Fax: +86 85115988

Chengshuo Wang, M.D., Ph.D.
Beijing TongRen Hospital, Capital Medical University
No. 1, DongJiaoMinXiang, DongCheng District, Beijing, China

E-mail: wangcs830@126.com

Mobile phone: +86 13911623569

Fax: +86 85115988

**Funding:** This work was supported by grants from the program for Changjiang scholars and innovative research team (IRT13082), the national natural science foundation of China (81420108009, 81630023, 81400444 and 81470678), Beijing Municipal Administration of Hospitals Clinical Medicine Development of Special Funding Support (ZYLX201310), Beijing natural science foundation (7131006), Beijing health bureau program for high level talents (2011-3-043), Beijing Municipal Administration of Hospitals’ Mission Plan (SML20150203), and the priming scientific research foundation for the senior researcher in Beijing TongRen Hospital (2017-YJJ-GGL-005). This work was supported in part by National Institutes of Health (NIH, http://www.nih.gov/) grant 1R01 HL083218 (LX). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Disclosure of conflict of interest:** None

**Word count:** 5007

**Author Contributions:** Bing Yan, Yang Wang and Ying Li performed cell culture, flow cytometry, immunohistochemistry, *in situ* hybridization, ELISA, Luminex and PCR assay. Bing Yan analyzed data and prepared manuscript. Lei Xiao, Hongfei Lou, Yifan Meng, Sihan Qi and Ming Wang participated in tissue sample collection and data discussion. Chengshuo Wang and Luo Zhang designed the study, analyzed data and prepared the manuscript.
ABSTRACT

Background: The interaction between epithelial cells and immune cells plays an important role in the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP); however, the mechanism/s underlying TH-biased inflammation in this process is/are largely unknown. Profiling protein expression in CRSwNP by shotgun proteomics suggested that Cystatin SN (CST1), a type 2 cysteine protease inhibitor, may play a role, as this was expressed with greatest difference in eosinophilic (E)CRSwNP and nonECRSwNP patients.

Objectives: To investigate the expression and role of CST1 in modulating eosinophilic inflammation in CRSwNP.

Methods: Sinonasal tissues were collected from 192 ECRSwNP, 52 nonECRSwNP and 40 control subjects. CST1 mRNA expression, localization, and concentration in the tissues were measured by real-time PCR, in situ hybridization, immunohistochemistry, and Elisa assay. Recombinant Cystatin SN was used to further explore the function of the molecule in dispersed nasal polyp cells and eosinophils extracted from polyp tissues and peripheral blood.

Results: CST1 was mainly expressed by epithelial cells, and significantly increased in ECRSwNP, but decreased in nonECRSwNP, compared with control subjects. CST1 expression was further increased in ECRSwNP with comorbid asthma and correlated with percentage of eosinophils in tissue samples. CST1 was induced by IL-4 and IL-13 in both ECRSwNP and nonECRSwNP tissue, and repressed by IL-17A in nonECRSwNP in the presence of neutrophils. CST1 enhanced eosinophil activation and recruitment by induction of interleukin-5 (IL-5).

Conclusion: Epithelium-derived CST1 modulates eosinophil activation and recruitment, the expression of which could be regulated by TH2 and TH17 cytokines.

Clinical implication: CST1 maybe a valuable biomarker and a potential therapeutic target for management of CRSwNP.
Capsule summary

This study reveals that TH2 and TH17 cytokines can regulate epithelium-derived CST1 expression. CST1 activates eosinophils and promotes their infiltration via IL-5-mediated pathway. CST1 represents a new promising target for the treatment of ECRSwNP.

Keywords: Chronic rhinosinusitis with nasal polyps (CRSwNP); CST1; Cystatin SN; eosinophil activation; eosinophil recruitment; epithelial cells; interleukin-5
Abbreviations

CD: cluster of differentiation (marker)
CRS: chronic rhinosinusitis
CRSsNP: chronic rhinosinusitis without nasal polyps
CRSwNP: chronic rhinosinusitis with nasal polyps
CST1: cystatin SN
CSTA: cystatin A
CSTF: cystatin F
DNPC: dispersed nasal polyp cells
ECP: eosinophil cationic protein
ECRSwNP: eosinophilic chronic rhinosinusitis with nasal polyps
EDN: eosinophil derived neurotoxin
ELISA: enzyme-linked immunosorbent assay
EPX: eosinophil peroxidase
FENO: fractional exhaled nitric oxide
GAPDH: glyceraldehyde–3–phosphate dehydrogenase
GM-CSF: granulocyte–macrophage colony stimulating factor
H&E: hematoxylin and eosin
HNEC: human nasal epithelial cells
IFN: interferon
IHC: immunohistochemistry
ILC2: group II innate lymphoid cells
IL: interleukin
ISH: in situ hybridization
nonECRSwNP: noneosinophilic chronic rhinosinusitis with nasal polyps
103 PMD: piecemeal degranulation
104 TH1 cells: T-helper 1 cells
105 TH17 cells: T-helper 17 cells
106 TH2 cells: T-helper 2 cells
107 TNF: tumor necrosis factor
INTRODUCTION

Chronic rhinosinusitis (CRS) is an inflammatory disease, with high prevalence worldwide [1]. CRS can be divided into CRS without nasal polyps (CRSsNP) and CRS with nasal polyps (CRSwNP), of which the latter type accounts for approximately 20%-25% of CRS, and is associated with a higher recurrence after surgical or pharmaceutical treatment [2]. CRSwNP can further be classified into two distinct immunohistological subtypes: eosinophilic CRSwNP (ECRSwNP) and noneosinophilic CRSwNP (nonECRSwNP) based on the observed eosinophil infiltration [3]. The two subtypes are also characterized by differences in TH type inflammation [1, 4-7]. ECRSwNP is pronounced as TH2-skewed and with relatively high recurrence and asthma co-morbidity rate; whereas nonECRSwNP is characterized by a TH1 or TH17 milieu and a lower recurrence and asthma co-morbidity rate [4, 5, 8, 9]. The two subtypes also exhibit significant differences in terms of geographic and racial distribution. In particular, studies have demonstrated that while ECRSwNP is clinically more predominant in CRSwNP patients in Europe and the United States, nonECRSwNP is more common in Asian patients [1].

Some studies have suggested that epithelial cells may also play a role in the pathogenesis of CRSwNP [10, 11]. Although different TH-biased inflammatory pathways have been well established in CRSwNP, relatively little is known about the influence of different TH status on the expression of distinct epithelial pathways or mediator/s responsible for the crosstalk between the epithelium and the immune cells in the ECRSwNP and nonECRSwNP inflammatory sub-types. In this regard, preliminary studies involving assessment of samples from subjects diagnosed as ECRSwNP and nonECRSwNP patients, and healthy controls, subjected to shotgun proteomics, followed by selection of specific proteins using "COMPARTMENTS" and “UniProt” database [12, 13], and analysis of mRNA level of each protein indicated that the difference in expression of Cystatin SN (CST1), was the most
significant between ECRSwNP and nonECRSwNP patients.

CST1 is a member of the type 2 cystatin proteins superfamily. Recent studies have proposed that the members of the cystatin superfamily may be involved in a number of immunologic processes [14-17]. However, the function of CST1 in the immunology processes is not fully understood. CST1 could inhibit the catalytic activity of clan C1 members such as cathepsins B, H, L, and S [17]. It is reported that CST1 is increased in childhood respiratory allergy and seasonal allergic rhinitis [18, 19]. In the latter disease, CST1 shows the inhibition of histamine release. However, to our knowledge, very few studies have explored the function of CST1 in CRSwNP.

The aim of the present study was therefore to investigate the expression of CST1 and its role in CRSwNP.
METHODS

Subjects and specimens

A total of 284 subjects, including 192 patients with ECRSwNP, 52 patients with nonECRSwNP and 40 healthy controls were enrolled in the study. The diagnosis of CRSwNP was based on standard criteria issued in the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 guidelines (EPOS 2012) [20]. The definition of ECRSwNP and nonECRSwNP was according to the criteria previously reported [4]. Patients with immunodeficiency, coagulation disorder, fungal sinusitis or cystic fibrosis, and pregnant women, did not participate in the study. None of the enrolled subjects had a history of aspirin sensitivity. Subjects undergoing septoplasty because of anatomic variations and without other sinonasal diseases were enrolled as control subjects. Polyp tissues from CRSwNP patients and inferior turbinate or uncinate mucosal tissues from control subjects were collected as biopsies or during surgery. Detailed information of subjects’ characteristics is provided in the Online Repository for this article as Table E1 and Figure E1.

This study was approved by the Ethics Committee of Beijing TongRen Hospital, Capital Medical University and written informed consent was obtained from each patient prior to enrollment.

Clinical Assessment

The diagnosis of asthma, allergic rhinitis or atopy, routine hematological parameters and the measurement of lower airway fractional exhaled nitric oxide (FENO), a biomarker of eosinophilic inflammation, were performed as previously described [9]. Detailed information of these procedures is provided in the Online Repository for this article.

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC)

H&E and IHC staining were performed as previously described [7, 21] to determine the
inflammation type and the location of CST1 in the tissue. Detailed information is provided in the Online Repository for this article as Methods section and Table E2.

Assessment of protein profiles of ECRSwNP, nonECRSwNP and control tissues using shotgun proteomics and secreted protein selection

Total proteins from 10 pooled samples each from ECRSwNP, nonECRSwNP and control subjects were isolated and processed further for quality control. A minimum of 2 unique, non-redundant peptides per protein was employed as the criteria for positive protein identification. Detailed information of these procedures is provided in the Online Repository for this article.

Differential proteins were subjected to “COMPARTMENTS” (https://compartments.jensenlab.org) and “UniProt” (www.uniprot.org) database for subcellular localization and secreted proteins acquisition [12, 13]. Detailed information on these procedures is provided in the Online Repository for this article.

RNA isolation, reverse transcription, and real-time PCR

Total RNA from tissue samples and cultured cells extracted with RNA extraction kit (TaKaRa Biotechnology, Dalian, China). The quality of total RNA was assessed with Nanodrop-2000 (Thermo Fisher Scientific, Waltham, MA, USA) and single-stranded cDNA was synthesized with PrimeScript™ RT Master Mix (TaKaRa Biotechnology). Real-time PCR was then performed with a SYBR Green method (TaKaRa Biotechnology) to assess the mRNA level of CST1 or mRNA coding eosinophilic granule proteins. Detailed information of the protocols and primer sequence are provided in the Online Repository for this article as Methods section and Table E3.

Assessment of CST1 and eosinophilic proteins by Elisa assay

The concentration of Cystatin SN in tissue sample or epithelial cells was measured using a
commercially available Elisa kit (Sigma-Aldrich, St. Louis, MO, USA). Similarly, eosinophilic cationic protein (ECP) and eosinophil peroxidase (EPX) levels released from eosinophils in the supernatant were determined using specific Elisa kits obtained from Cusabio Life Sciences, Wuhan, China. Detailed information on these procedures is provided in the Online Repository for this article as Methods section and Table E4.

Human nasal epithelial cells (HNECs) culture

HNECs were prepared from nasal polyps or healthy mucosa, following enzymatic digestion of the tissue, and purified HNECs were cultured using an air-liquid interface (ALI) method [22]. Cell cultures were treated with several mediators; including cathepsin (CTS)B, CTSC, IL-3, IL-4, IL-5, CXCL8, IL-13, IL-10, IL-17A, GM-CSF, and IFN-γ; for 24 hours and then harvested for assessment of CST1 mRNA by quantitative RT-PCR as described above. Detailed information on these procedures is provided in the Online Repository for this article.

Assessment of cytokines using Luminex system

The concentration of cytokines, including IL-5, IL-17A, IFN-γ, GM-CSF, CXCL8 and TNF-α in tissue samples and IL-4, IL-5, IL-10, IL-13, IL-17A, IL-1β, IFN-γ, CCL5, CCL11, CCL24, CCL26 and GM-CSF in cultured cells was determined using Luminex assay. Specific details of the cytokines analyzed are provided in the Online Repository for this article as Methods section and Table E4.

Assessment of CST1 mRNA transcriptional site by in situ hybridization (ISH)

CST1 mRNA transcriptional site in tissue sections of ECRSwNP samples was analyzed by ISH. Detailed information of the procedure is provided in the Online Repository for this article.

Eosinophil isolation and culture

Peripheral blood eosinophils were purified using eosinophil isolation kit (MiltenyiBiotec, San
Diego, CA, USA). Eosinophil purity was assayed by flow cytometry (Figure E5). Isolated eosinophils were cultured in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and stimulated with recombinant Cystatin SN, IL-5, GM-CSF (R&D Systems) or E64d (Cayman Chemical, Michigan, USA) for 24-hours. At the end of this incubation the eosinophils were harvested and investigated further by quantitative RT-PCR for expression of ECP, EPX and PU.1 mRNAs; by Elisa assay for the concentration of ECP and EPX in the supernatants; by Luminex assay for synthesis of IL-4, IL-5, IL-10, IL-13, IL-17A, IL-1β, IFN-γ, CCL5, CCL11, CCL24, CCL26 and GM-CSF; and by flow cytometry for CD69 expression. Proliferation was determined up to 48-hours. Detailed information on this protocol and procedures is provided in the Online Repository for this article.

**Flow cytometry**

White blood cells were stained for APC-conjugated CST1 antibody under the baseline and IL-4 stimulation. Isolated eosinophils were stained for Alexa Fluor 700-conjugated Siglec-8 for purity detection or FITC-conjugated CD69 antibody for activation after treatment with recombinant Cystatin SN (R&D Systems) or E64d (Cayman Chemical). Then the cells were subjected to flow cytometry. Detailed information of the procedures employed is provided in this article’s Online Repository, and the antibodies employed by flow cytometry were shown in Table E2.

**Dispersed nasal polyp cells (DNPC) culture**

DNPCs were cultured and treated with recombinant Cystatin SN (R&D Systems) and E64d (Cayman Chemical) for 24-hours. At the end of this incubation, the cells and cell-free supernatants were harvested for further analysis of determination mRNA level of ECP, EPX and PU.1 and for the detection of cytokines. Detailed information of these procedures is
provided in the Online Repository for this article.

**Eosinophil migration assay**

Eosinophils isolated from peripheral blood of subjects were added into the upper compartments of transwells at a number of $5 \times 10^5$ in 200 µL RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific). The lower chambers were added with the same medium containing 100ng/mL recombinant Cystatin SN or 10 µM E64d. The cells were incubated at 37℃ and 5% CO₂ for 1-4 hours. At the end of incubation, the medium in lower chambers was collected and assessed for the number of eosinophils present. Detailed information of the assay is provided in the Online Repository for this article.

**Eosinophil recruitment**

Polyp tissues were rinsed in PBS containing 1% penicillin, 1% streptomycin, and 1% amphotericin, then weighed and cut into smaller sections about 0.1g in weight. 10 µL of 10 µg/mL recombinant Cystatin SN, 10 µg/mL recombinant IL-5, 10 µg/mL recombinant GM-CSF, 10 µg/mL recombinant CCL24, 1 mM E64d or PBS were injected separately or together with 10 µL of 10 µg/mL IL-5 blocking antibody into each section and co-cultured with WBCs ($10^6$ cells in each well) for 96 hours, for assessment of eosinophils infiltrating the tissue. Detailed information of the assay is provided in the Online Repository for this article as Methods section and Figure E9.

**Statistical analysis**

All statistical analysis was performed using SPSS version 23.0 (IBM Corp., Armonk, NY, USA). All data are presented as mean ± SEM unless otherwise noted. Differences between groups were analyzed using the Kruskal-Wallis ANOVA testing. Pearson's chi-squared test ($\chi^2$) or Fisher’s exact test was applied to sets of categorical data. Correlations were assessed
using the Spearman correlation, and defined as strong when R value was >0.7, moderate when in the 0.5-0.69 range, or weak when in the 0.3-0.49 range. Paired \( t \)-test was used to test the significance of cell culture data. Samples pre and post glucocorticoid treatment were compared by paired \( t \)-test. Cells treated with recombinant CST1, E64d and/or IL-5 neutralizing antibody was analyzed by two-way ANOVA testing. \( P<0.05 \) was considered statistically significant. All significance levels presented are two-tailed.
RESULTS

Protein profiles and screening of the secreted proteins in nasal polyps tissue

Polyp tissues from patients with ECRSwNP, nonECRSwNP and mucosa from healthy controls subjected to shotgun proteomics demonstrated a total of 1022, 872, and 944 proteins, respectively. To characterize inflammatory types present among these groups, IL-5, IL-17A, IFN-γ, GM-CSF, TNF-α and CXCL8 (IL-8) were assessed in appreciable quantities, of which the levels of IL-5, GM-CSF and IFN-γ were significantly higher in ECRSwNP tissue compared to control; while CXCL8, IL-17A, and IFN-γ were significantly higher in nonECRSwNP tissue compared to control (Figure E1).

Assessment of secreted proteins in nasal polyp tissues by COMPARTMENTS and UniProt databases followed by RT-PCR: to assess the gene/s expressing secreted proteins present exclusively in one group, or expressed at least five-fold higher between at least two groups: demonstrated that CST1 was expressed with the greatest difference between ECRSwNP and nonECRSwNP.

CST1 expression in CRSwNP

Assessment of the expression of CST1 relative to GAPDH (housekeeping gene), indicated that the CST1 mRNA levels were significantly increased in ECRSwNP tissues compared with nonECRSsNP ($P < 0.001$) or control ($P < 0.001$) tissues (Figure 1A). Furthermore, CST1 mRNA levels were significantly decreased in nonECRSwNP tissues compared with controls ($P < 0.05$) (Figure 1A). We further sub-classified ECRSwNP patients into asthmatic and non-asthmatic subjects, and found that the asthmatics demonstrated even higher levels of CST1 mRNA compared with the non-asthmatics ($P < 0.01$) (Figure 1B). Measurement of the concentration of CST1 protein by Elisa demonstrated that Cystatin SN levels were significantly increased in ECRSwNP ($P < 0.001$) and decreased in nonECRSwNP ($P < 0.05$) compared with control subjects (Figure 1C). Similarly, Cystatin SN levels were significantly
increased in asthmatic ECRSwNP patients, compared with non-asthmatic ECRSwNP patients ($P < 0.01$; Figure 1D); thus confirming the findings for increased expression of CST1 mRNA in ECRSwNP patients.

Cystatin SN is mainly expressed in nasal epithelial cells

Immunohistochemical (IHC) staining of tissues from the three groups of subjects indicated that Cystatin SN was localized mainly to the epithelium (Figure 2A). Furthermore, semi-quantitative analysis of the staining indicated that consistent with the results obtained using the Elisa assay, Cystatin SN levels were significantly increased in ECRSwNP ($P < 0.001$) and decreased in nonECRSwNP ($P < 0.05$), compared with control subjects (Figure 2B). ISH for determination of CST1 mRNA transcriptional site further confirmed the epithelial cells to be the major site in this regard (Figure 2C).

Flow cytometric assessment to detect the potential expression of CST1 in hematopoietic cells indicated that very low level of CST1 was expressed in these cells, both at baseline and following stimulation with IL-4 (Figure E2).

Correlation of Clinical Parameters with CST1 mRNA and protein levels

For ECRSwNP patients, both tissue CST1 mRNA and protein levels were significantly, but weakly, correlated positively with percentage of eosinophils infiltrating the tissues ($P = 0.003$, $R = 0.443$ for mRNA, $P = 0.026$, $R = 0.335$ for protein) and fractional exhaled nitric oxide (FENO; $P = 0.013$, $R = 0.411$ for mRNA, $P = 0.005$, $R = 0.461$ for protein) (Figure 3). In contrast, no significant correlation was observed between CST1 mRNA or protein levels and percentage of peripheral blood eosinophils ($P = 0.382$ for mRNA, $P = 0.068$ for protein).

Regulation of CST1 in CRSwNP

We explored the factors to regulate the production of CST1. As cathepsin family members act as the substrate for CST1, we assessed the expression of these proteins by data mining of
microarray, RNAseq, and our proteomic data and found that two members of the family, Cathepsins B and C were upregulated in ECRSwNP (Table E5). Thus, we further assessed the function of cathepsins B and C in the CST1 expression. In this regard, recombinant cathepsins B and C were not able to induce CST1 expression in epithelial cells (Figure E3).

Next typical TH cytokines which impact the expression of CST1 were assessed, and in accordance with previous reports [18, 19], we found that IL-4 induced CST1 expression (Figure 4). IL-13 also induced CST1 production, but not to the same level as IL-4. In contrast, IL-17A significantly depressed the expression of CST1, in polyps infiltrated with neutrophils, but not in ECRSwNP polyps (Figure 4). However, IL-3, IL-5, IL-8, IL-10, GM-CSF, and IFN-γ had little effect on CST1 (Figure E3).

**CST1 activates eosinophils**

The positive correlation noted in polyp tissue for CST1 expression and the percentage of eosinophil suggested that CST1 may affect eosinophils. In this regard, incubation of DNPC for 24 hours with recombinant Cystatin SN or E64d, a chemical cysteine protease inhibitor which can mimic the activity of Cystatins [23], demonstrated that both these treatments significantly increased the mRNA levels of EPX and ECP (Figure E4). While EPX and ECP proteins were also detected in the medium, the levels of these proteins were lower than the limit of detection in several samples, and thus these data were not available. However, incubation of eosinophils isolated from peripheral blood of patients with ECRSwNP with recombinant Cystatin SN or E64d demonstrated that consistent with the results from studies employing DPNC, the levels of mRNAs for EPX and ECP were significantly increased after recombinant Cystatin SN or E64d treatment for 24 hours in comparison with control medium (mock)(Figure 5A). Furthermore, the levels of EPX and ECP proteins were also significantly increased after recombinant Cystatin SN treatment for 24 hours (Figure 5B). Similarly,
assessment of the expression of CD69 on eosinophils, a protein increased during eosinophil activation and necessary for degranulation, demonstrated that both recombinant Cystatin SN and E64d significantly enhanced the number of CD69+ eosinophils (Figure 5C). The purity of the blood eosinophils preparation was assessed by flow cytometry and found to be 93.4% (Figure E5). Eosinophils isolated from several patients were also treated with recombinant IL-5 or GM-CSF as positive control. (Figure E6).

As activated eosinophils secrete granule contents by a mechanism termed piecemeal degranulation (PMD), which ensures that the cells do not die after degranulation and can produce more granules [24], we assessed the mechanism by which CST1 might promote the synthesis of eosinophils granule; i.e. by enhancing the proliferation eosinophils or by stimulating synthesis. Assessment of the numbers of cultured eosinophils incubated with recombinant Cystatin SN and E64d demonstrated that the eosinophil numbers were not significantly greater after 48 hours compared to controls (Figure 5D); suggesting that the CST1 may enhance granule synthesis of “resident eosinophils”. As PU.1, a transcriptional factor, has been reported to be crucial for the synthesis of eosinophil granule proteins such as ECP and EPX [25], we also assessed the effect of recombinant Cystatin SN and E64d on the expression of PU.1, and demonstrated that this was also significantly increased by Cystatin SN and E64d compared with control medium (Figure 5A).

**CST1 promotes eosinophil recruitment**

Assessment of the effect of CST1 on eosinophil recruitment using transwells with eosinophils treated with recombinant Cystatin SN or E64d up to four hours demonstrated no significant increase in the numbers of migrating eosinophils, compared with controls (Figure E7). However, polyps injected with recombinant Cystatin SN or E64d and cultured with the white blood cells of the same individual from whom the polyps were obtained, demonstrated that after 96-hours large numbers of eosinophils were present in tissue sections treated with
recombinant Cystatin SN and E64d, compared with PBS (Figure 6, E8 and E9). Injection of recombinant IL-5, GM-CSF and CCL24 (Eotaxin-2), as positive controls, also demonstrated significantly increased numbers of eosinophils compared to PBS, and thus confirmed the effects of CST1, as well as the validity of the model in assessing the efficacy of the specific mediators in the recruitment of eosinophils (Figure E8). This finding suggests that the recruitment of eosinophils by CST1 is unlikely to be due to ligand or receptor binding on the eosinophils, but is probably mediated via cytokines or molecules.

**CST1 enhances interleukin (IL)-5 expression**

As previous studies have indicated that CST1 mainly plays a role in eosinophilic inflammation via some mediators, to illustrate the whole vision of the inflammation bias, we assessed the effect of CST1 on the induction or inhibition of a variety of TH cytokines, including IL-4, IL-5, IL-10, IL-13, IL-17A, IL-1β, and IFN-γ. Incubation of dispersed nasal polyp cells (DNPC) from ECRSwNP tissues and eosinophils isolated from ECRSwNP patients with both recombinant Cystatin SN and E64d for 24 hours significantly upregulated the expression of IL-5 in both DNPC and eosinophils (Figure 7). IL-17A and IFN-γ were not detectable in the eosinophils using the Luminex system in the present study.

We further explored the effect of CST1 on the concentrations of CCL5, CCL11, CCL24, CCL26 and GM-CSF; which are also known to be effective mediators in the recruitment and activation of eosinophils. Our study demonstrated that incubation of eosinophils isolated from ECRSwNP patients with recombinant Cystatin SN did not significantly alter the concentrations of CCL5, CCL11, CCL24, CCL26 released from eosinophils; whereas the concentration of GM-CSF was below the detection limit for the assay (Figure E10).

**IL-5 is involved in CST1 induced eosinophilic inflammation**
To determine the role of IL-5 in the function of CST1, eosinophils were pre-treated with medium containing 10µg/mL IL-5 blocking antibody (R&D systems) or with medium alone for one hour, and then incubated for a further 24 hours with recombinant Cystatin SN or E64d. Both Cystatin SN and E64d significantly increased the expression of EPX, ECP, and PU.1 mRNA (Figure 8A) and release of EPX and ECP protein in the cell culture media (Figure 8B) in the absence of IL-5 blocking antibody. However, these Cystatin SN- and E64d-induced increases were abolished by IL-5 blocking antibody (Figure 8A and 8B, respectively).

Similarly, to determine whether IL-5 blocking antibody could decrease CST1- and E64d-induced infiltration of eosinophils, polyps tissues were injected with PBS, recombinant Cystatin SN or E64d in the absence or presence of IL-5 blocking antibody. Similar to the findings for EPX, ECP, and PU.1, recombinant Cystatin SN or E64d significantly increased eosinophil numbers infiltrating the polyp tissues compared with mock PBS, and this increase in eosinophils was significantly attenuated in the presence of IL-5 blocking antibody (Figures 8C and 8D).

**Glucocorticoids partially suppress the expression of epithelial CST1**

We investigated the effect of glucocorticoids on epithelial CST1 mRNA and protein expression. Samples were acquired pre- and post-treatment by biopsy and following surgery. Patients underwent a 2-week oral corticosteroid therapy (methylprednisolone at 24 mg once daily during 7 days followed by 4 mg reduction every other day during the other 7 days) [26, 27]. This study demonstrated that glucocorticoid significantly suppressed both CST1 mRNA and protein expression compared to baseline. However, regular doses of glucocorticoids could not attenuate CST1 expression in ECRSwNP to the level of normal controls, and even post-treatment, the CST1 expression on epithelial cells of patients with ECRSwNP was...
significantly higher compared to healthy controls (Figure 9).
DISCUSSION

Research into CRSwNP over the last few decades has shown that this is a complex and heterogeneous disease [1]. A recent study has indicated that several clusters of CRSwNP can be identified based on distinct clinical pathobiological features and differences in response to treatment [7, 28-31]. Immunohistologically, CRSwNP can be divided into ECRSwNP and nonECRSwNP [3, 7]; with ECRSwNP predominating in Caucasian patients demonstrating Th2-biased cytokine profiles, and nonECRSwNP predominating in mostly Asian patients demonstrating Th1/Th17-biased patterns [4-6]. Furthermore, ECRSwNP is associated with a higher likelihood of comorbid asthma, and thus, may be further divided into the subtypes according to asthma comorbidity or not. As a clustering axis extending from nonECRSwNP to ECRSwNP without asthma to ECRSwNP with comorbid asthma, the peripheral blood eosinophil counts and percentage eosinophils are generally increased, suggesting that the probability of the disease to be a systemic disorder is elevated. For the purpose of treatment, confirming the subtype of the disease is possibly the first and most important thing, because this helps in deciding the strategy for the route of administration, as either local or systemic treatment, in minimizing the systemic risks and reducing the drug load as well as the costs. Hence, determining an effective biomarker for the clustering is likely to aid in the diagnosis and appropriate treatment for CRSwNP.

The present study has indicated that CST1 (Cystatin SN) may be a novel useful biomarker for ECRSwNP, because this was significantly increased in tissue from patients with ECRSwNP and decreased in tissues of patients with nonECRSwNP compared with inferior turbinate or uncinate tissue from control subjects. Furthermore, the expression of CST1 was significantly increased in ECRSwNP patients with comorbid asthma compared to ECRSwNP patients without asthma. Indeed, the levels of both CST1 mRNA and protein were also found to be positively correlated with the percentage of tissue eosinophils and with
FENO level in ECRSwNP patients. Similar to our findings, Kato and colleagues [32] have recently demonstrated that the expression of CST1 was significantly elevated in ECRSwNP compared to nonECRSwNP patients. Collectively, these findings suggest that CST1 may serve as a reliable biomarker for ECRSwNP.

Activation and recruitment of eosinophils are major features of eosinophilic inflammation, and in this regard it is likely that CST1 plays a role in both processes. Activated eosinophils undergo degranulation, releasing proteins such as EPX, ECP, EDN, etc, which can be toxic to invading cells and also be responsible for tissue damage and remodeling. It has been reported that the apoptosis of eosinophils is delayed in CRSwNP, and the infiltrating eosinophils release the granules without undergoing self-destruction [33]. Although different mechanisms of eosinophil degranulation have been postulated [33], the mechanisms underlying the regeneration/sustainability of granules/granule proteins in activated eosinophils are presently not fully understood. Findings from the present study suggest that epithelial cell-derived CST1 promotes eosinophil granule protein synthesis by inducing IL-5. Although several cells including Th2 and ILC2 could be the source of IL-5, our study has further demonstrated that CST1 also enhances the production of IL-5 in eosinophils. Thus, it is possible that CST1-induced IL-5 synthesis in eosinophils may be involved in autocrine regulation of granule protein synthesis. Furthermore, the present study has demonstrated that CST1-mediated activation and recruitment of eosinophils via IL-5 may be reciprocated by IL-4 or IL-13 released from eosinophils.

Our study has further suggested that Cystatin SN expressed in CRSwNP tissue also has the potential to enhance the recruitment and infiltration of eosinophils directly from the blood via involvement of IL-5 and activation of eosinophils involving degranulation. The infiltration of eosinophils was significantly enhanced by the treatment of recombinant Cystatin SN or E64d for 96 hours, which reflects the promotion of eosinophilic inflammation
of CST1 in vivo. Overall, these data indicate that CST1 is involved in the activation of existing eosinophils in the tissue and is responsible for eosinophil recruitment under the Th2 micro-environments. Furthermore, the finding from the present study that recombinant Cystatin SN did not recruit eosinophils effectively over a relatively short period of four hours, but significantly increased eosinophil recruitment over a longer period of 96 hours suggests that Cystatin SN is unlikely to affect eosinophils directly by binding to specific receptor/s on the surface of eosinophils. Indeed, although eosinophils are known to express numerous cell surface receptors; including chemokine receptors, Fc receptors, pattern recognition receptors, lipid mediator receptors, cytokine receptors, complement receptors, and adhesion receptors [35]; to our knowledge presently there are no reports of cystatin receptors on eosinophils.

Studies in cystatin F (CSTF) knock-out mice have reported that eosinophils from these mice had reduced lifespan, reduced granularity, and disturbed granule morphology [34]. Furthermore, cysteine protease inhibitors restored granularity, demonstrating that control of cysteine protease activity by CSTF was critical for normal eosinophil development. However, microarray, RNAseq and proteomics assessment has not detected the expression of either CSTF mRNA or protein in human CRSwNP patients; whereas CST1, CST2, CSTA, CSTB, and CSTC have been shown to be present [34]. RNAseq assay indicated that CST1 was upregulated in ECRSwNP compared with healthy control; the fold change of CST1 for ECRSwNP vs. CTRL is 1732.2; which also indicated that CSTA and CSTB were upregulated in both ECRSwNP and nonECRSwNP compared with healthy control; with no significant difference between the two subtypes; the fold change of CSTA for ECRSwNP vs. CTRL is 4.32 and for nonECRSwNP vs. CTRL is 3.95; the fold change of CSTB for ECRSwNP vs. CTRL is 1.34 and for nonECRSwNP vs. CTRL is 2.28 [35]. Proteomics demonstrated CSTB and CSTC to be present in the nonECRSwNP group but were not be detectable in control and ECRSwNP (unpublished data). Similarly, microarray analysis demonstrated CST1, CSTA and
CST2 to be increased in CRSwNP; however, the expression of CSTA and CST2 is relatively low compared with that of CST1. The fold change of CST1, CSTA and CST2 for CRSwNP versus control is 31.53, 2.1 and 8.34 respectively [36, 37]. Thus, unlike the internal signaling of CSTF for mice, the CST1 mediated external signaling might be the way to regulate eosinophil by Cystatins in humans and reflects the interaction between epithelium and eosinophils.

The present study showed that CST1 could be regulated by a variety of cytokines, including IL-4 and IL-13 which promoted the transcriptional level of CST1 in epithelial cells, whereas IL-17A decreased its expression. Interestingly, as this decrease could only be observed in neutrophil infiltrated polyps, it may reflect that IL-17A does not directly regulate CST1 but acts via another molecule, which is absent or undetectable in normal or eosinophilic CRSwNP. These results nevertheless further support the concept that CRSwNP is a heterogeneous condition and each subtype presents with the distinct genetic background. However, as activated eosinophils could also release IL-4, IL-5 and IL-13, this suggests that there may be a CST1-mediated positive feedback loop that propagates eosinophil recruitment, activation and synthesis of eosinophil granular proteins in ECRSwNP (Figure E11).

Thus, CST1 may be an effective biomarker for rapid detection of CRSwNP subtypes for several reasons. First, the threshold window is wide enough that the mRNA relative expression ratio of typical ECRSwNP to nonECRSwNP is as high as hundreds-fold. Second, the absolute expression of CST1 is high in ECRSwNP; which is as high as or even higher than the expression of housekeeping genes such as GAPDH. The wide threshold window and the large amount of CST1 in ECRSwNP make it easy for clinical examination. Third, our research provides positive correlation of CST1 expression and the percentage of eosinophils, which could be the basis for the biomarker development.

Taken together, these data indicate that CST1 participates in eosinophilia and the cysteine
protease inhibitor ability is required, particularly as tissue levels of CST1 were profoundly
increased in ECRSwNP tissue and decreased in nonECRSwNP. This difference in expression
results from the different TH type of inflammation. Epithelial cells are the major CST1
producing cell in ECRSwNP and the expression of CST1 is positively correlated with the
percentage of tissue eosinophils and FENO level. Overproduction of CST1 may lead to
eosinophil granule protein synthesis and recruitment of eosinophils by IL-5. Collectively,
these findings suggest that CST1 is a valuable biomarker for systemic CRSwNP, and
targeting CST1 might be of therapeutic value in treating patients with TH2 dominated
CRSwNP.
REFERENCES


FIGURE LEGENDS

FIGURE 1. The expression of CST1 in nasal tissue from CRSwNP patients. A, CST1 mRNA levels in tissues from control subjects, ECRSwNP patients and nonECRSwNP patients; B, CST1 mRNA levels in tissues from control subjects and ECRSwNP patients with or without asthma; C, CST1 protein levels in tissues from controls subjects, ECRSwNP and nonECRSwNP patients; D, CST1 protein levels in tissues from control subjects and ECRSwNP patients with or without asthma.

FIGURE 2. Localization of CST1 in nasal tissue. A, representative figures showing CST1 staining of nasal mucosa or polyps tissue from control subjects (CTRL), ECRSwNP patients and nonECRSwNP patients. Bars= 20 µm (400× magnification); B, semi-quantitative analysis of stained epithelial cells in CTRL, ECRSwNP and nonECRSwNP patients (for each group, n= 5); C, in situ hybridization of CST1 mRNA in ECRSwNP tissue. Purple color represents positive expression of CST1. Bars= 20 µm.

FIGURE 3. Correlation of CST1 expression with clinical index in ECRSwNP patients. A-B, correlation of CST1 mRNA level with the percentage of tissue eosinophils and FENO level, respectively. C-D, correlation of CST1 protein level with the percentage of tissue eosinophils and FENO level, respectively.

FIGURE 4. Regulation of CST1. A, representative figures showing hematoxylin and eosin (H&E) staining for each type of CRSwNP. Bars= 20 µm (400× magnification); and B, mRNA and C, protein levels of CST1 measured in epithelial cells cultured from nasal mucosa from control subjects or polyp tissues stimulated with IL-4, IL-13 and IL-17A.

FIGURE 5. The effect of CST1 on the activation of eosinophils isolated from peripheral blood of patients with ECRSwNP. Isolated eosinophil cultures were treated with 100ng/mL recombinant Cystatin SN or 10µM E64d for 24 hours (A-C) or 48 hours (D) and then assessed for A, the relative expression of ECP, EPX and PU.1 mRNAs in eosinophils and B,
concentrations of ECP and EPX released in the culture medium; C, CD69 expression in
eosinophils; D, proliferation of eosinophils. For A and B, samples from the same individual
are shown in the same color, and the mean values for each group are shown as green squares.

**FIGURE 6.** CST1 promotes recruitment of eosinophils. Nasal polyp sections were injected
with 10 µg/mL recombinant Cystatin SN or 1 mM E64d in the volume of 10 µL per 0.1g
tissue and then incubated with white blood cells isolated from the individual from whom the
polyp tissue was obtained. After incubation for 96-h, the polyp sections were collected and
following H & E staining were assessed for the number of eosinophils present. Bars= 50 µm
(200× magnification).

**FIGURE 7.** Effect of recombinant Cystatin SN and E64d on cytokine expression in nasal
polyps and eosinophils. A, dispersed nasal polyp cells (DNPC) and B, cultured eosinophils
were treated with 100 ng/mL recombinant Cystatin SN or 10 µM E64d for 24 hours, and the
cell-free supernatants were collected for assessment of cytokines by Luminex. *: P < 0.05; **:
P < 0.01; ***: P < 0.001.

**FIGURE 8.** Effect of IL-5 blocking antibody (10 µg/mL) on CST1 or E64d-induced A,
relative mRNA expression of ECP, EPX, and PU.1; B, ECP and EPX protein; and C-D,
recruitment of eosinophils. For A and B the mean value for each group is shown as the green
square; C shows representative images for recruitment of eosinophils induced by recombinant
Cystatin SN or E64d; Bars = 50 µm (200× magnification); D shows the average numbers of
eosinophils recruited in each group of five high power fields (HPF) at 400× magnification.

**FIGURE 9.** Effect of glucocorticoids on the expression of epithelial CST1. A and B,
representative CST1 staining in nasal tissue and quantitative analysis pre- and post-
glucocorticoids treatment (n=5). Bars= 20 µm (400× magnification). C, relative mRNA level
and protein expression of CST1 pre- and post-glucocorticoid treatment (n=10 and 11
respectively).
CST1 mRNA (CRSwNP)

- $P < 0.05$
- $P < 0.001$
- $P < 0.001$

CST1 $^{\text{Ago}}$ normalized to GAPDH (log10)

CTRL (n=20)  ECRSwNP (n=44)  nonECRSwNP (n=22)
CST1 mRNA (ECRSwNP)

\[ P < 0.001 \]
\[ P < 0.001 \]
\[ P < 0.01 \]

CST1 mRNA was measured and normalized to GAPDH. The data was compared between CTRL (n=20), asthmatic (n=19), non-asthmatic (n=25), and ECRSwNP groups. The results showed significant differences with p-values below 0.001 and 0.01.
Cystatin SN protein (CRSwNP)

\[ P < 0.05 \]

\[ P < 0.001 \]

\[ P < 0.001 \]

\( \mu g/g \) of tissue weight

CTRL (n=20)  ECRSwNP (n=44)  nonECRSwNP (n=22)
Cystatin SN protein (ECRSwNP)

\[
P < 0.001
\]
\[
P < 0.001 \quad P < 0.01
\]

µg/g of tissue weight

CTRL (n=20)  
asthmatic (n=19)  
non-asthmatic (n=25)

ECRSwNP
<table>
<thead>
<tr>
<th>CTRL</th>
<th>ECRSwNP</th>
<th>nonECRSwNP</th>
</tr>
</thead>
</table>


Cystatin SN protein (Tissue)

\[
\begin{align*}
&P < 0.05 \\
&P < 0.001 \\
&P < 0.001
\end{align*}
\]

Semi-quantitative analysis (Score)

CTRL (n=5)  ECRSwNP (n=5)  nonECRSwNP (n=5)
$P = 0.013 \quad R = 0.411$

CST1 mRNA normalized to GAPDH
CST1 mRNA (Epithelial cell)

- **CTRL** (n=5)
- **ECRSwNP** (n=5)
- **neu CRSwNP** (n=5)
- **non-neu CRSwNP** (n=5)

- **Mock**
- **IL-4 (100 ng/mL)**
- **IL-13 (100 ng/mL)**
- **IL-17A (100 ng/mL)**

Relative mRNA expression (Log10)

**Significance:**
- ***p < 0.001**
Proliferation rate (Eosinophils)

- Mock
- CST1 (100 ng/mL)
- E64d (10 μM)

Fold

0.0  0.5  1.0  1.5

0  24  48

Hours
Cytokines expression (DNPC supernatants)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Mock</th>
<th>CST1(100ng/mL)</th>
<th>E64d(10μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>IL-17A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
<td>**</td>
</tr>
</tbody>
</table>

Cytokines expression (pg/mL)
A graph showing the eosinophil number/HPF for different treatments:

- PBS
- CST1
- E64d

The graph shows statistical significance with:

- $P<0.01$

For IL-5 blocking Ab:

- (-) no significance
- (+) no significance

Legend:

- Red triangles represent PBS
- Black circles represent CST1
- Gray squares represent E64d
Cystatin SN

Pre-treatment

Post-treatment
Cystatin SN protein (Tissue)

$P < 0.05$

$P < 0.001$

$P < 0.001$

Semi-quantitative analysis (Score)

CTRL (n=5)  Pre (n=5)  Post (n=5)

Glucocorticoids treatment
CST1 mRNA (Tissue)

Cystatin SN protein (Tissue)

CTRL (n=20) Pre (n=10) Post (n=10)

CTRL (n=20) Pre (n=11) Post (n=11)

Glucocorticoids treatment

Glucocorticoids treatment
ECP (Culture medium)

- $P < 0.001$
- $P < 0.01$
- $P < 0.01$

EPX (Culture medium)

- $P < 0.01$
- $P < 0.05$
- $P < 0.01$
Epithelium-derived Cystatin-SN enhances eosinophil activation and infiltration via interleukin-5 in chronic rhinosinusitis with nasal polyps

Authors: Bing Yan, Ph.D.\textsuperscript{1,2}, Hongfei Lou, M.D., Ph.D.\textsuperscript{1}, Yang Wang, B.S.\textsuperscript{1,2}, Ying Li, B.S.\textsuperscript{1,2}, Yifan Meng, M.D., Ph.D.\textsuperscript{1}, Sihan Qi, M.D., \textsuperscript{1,2}, Ming Wang, Ph.D.\textsuperscript{1,2}, Lei Xiao, M.D., Ph.D.\textsuperscript{3}\textsuperscript{*}, Chengshuo Wang, M.D., Ph.D.\textsuperscript{1,2}*, Luo Zhang, M.D., Ph.D.\textsuperscript{1,2,4}\textsuperscript{*}

*: These authors contributed equally to the study.

1 Department of Otolaryngology, Head and Neck Surgery, Beijing TongRen Hospital, Capital Medical University, Beijing 100730, China.
2 Beijing Key Laboratory of Nasal Diseases, Beijing Institute of Otolaryngology, Beijing 100005, China.
3 Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA.
4 Department of Allergy, Beijing TongRen Hospital, Capital Medical University, Beijing 100730, China.

Corresponding authors:
Luo Zhang, M.D., Ph.D.,
Beijing TongRen Hospital, Capital Medical University
No. 1, DongJiaoMinXiang, DongCheng District, Beijing, China
E-mail: dr.luozhang@139.com
Mobile phone: +86 13910830399
Fax: +86 85115988

Chengshuo Wang, M.D., Ph.D.
Beijing TongRen Hospital, Capital Medical University
No. 1, DongJiaoMinXiang, DongCheng District, Beijing, China

E-mail: wangcs830@126.com

Mobile phone: +86 13911623569

Fax: +86 85115988
METHODS

Subjects and specimens

This study was approved by the Ethics Committee of Beijing TongRen Hospital, Capital Medical University. Written informed consent was obtained from each patient. In total, 284 subjects were enrolled, including 192 patients with ECRSwNP, 52 patients with nonECRSwNP and 40 healthy controls. The diagnosis of CRSwNP was based on standard criteria issued in the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 guidelines (EPOS 2012) [E1]. The definition of ECRSwNP and nonECRSwNP was according to the criteria previously reported [E2]. Patients with immunodeficiency, coagulation disorder, fungal sinusitis, cystic fibrosis, and pregnant women, did not participate in the study. None of the enrolled subjects had a history of aspirin sensitivity. Subjects without other sinonasal diseases and undergoing septoplasty because of anatomic variations were enrolled as control subjects. Polyp tissues from CRSwNP patients and inferior turbinate or uncinate mucosal tissues from control subjects were collected as biopsy or during surgery. Oral glucocorticoid, intranasal steroid spray and anti-leukotrienes were discontinued at least four weeks before biopsy and surgery. Detailed information of subjects’ characteristics is provided in Table E1 and Figure E1.

Clinical Assessment

Atopic status was evaluated based on ImmunoCAP to detect IgE antibodies against common inhalant allergens (Phadia, Uppsala, Sweden) (cutoff ≥ 0.7 kU/mL) [E3]. The diagnosis of asthma was based on the Global Initiative for Asthma 2006 guideline [E4]. The diagnosis of allergic rhinitis was based on the Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines-2016 revision [E5]. Oral fractional exhaled nitric oxide (FENO) was measured using the NIOX electrochemical analyzer (Aerocrine, Solna, Sweden) at a flow rate of 50 mL/s [E3]. Peripheral blood was collected before surgery, and cells were counted using an
Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC)

Tissue samples were fixed, dehydrated, infiltrated, and embedded in paraffin, and the tissue was sectioned at 5 µm thickness using a Leica RM2235 Cryostat (Leica Microsystems, Inc, Bannockburn, Ill). After deparaffinization and rehydration, sections were routinely stained with H&E.

IHC staining was performed as follows: Sections were rehydrated, repaired and endogenous peroxidase activity was blocked by 3% H₂O₂ and nonspecific binding was blocked with 3% goat serum in 0.3% Tween-20/PBS. Tissue sections were then incubated with rabbit anti-human CST1 mAb (Abcam, Cambridge, UK) in blocking buffer overnight at 4°C. Sections incubated with the same concentrations of control rabbit IgG (ZSGB-Bio, Beijing, China) served as control. Sections were rinsed and then incubated in HRP conjugated secondary goat anti-rabbit antibody (ZSGB-Bio) for 1 hour at room temperature. Sections were rinsed again and incubated in diaminobenzidine reagent (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature. Sections were then rinsed in dH₂O, counterstained with hematoxylin, dehydrated, cleared, mounted, and cover-slipped. Microscopic analysis was performed with Olympus BX51 microscope using ×20 or ×40 objective lens, and images were collected using DP2-BSW software (Olympus, Center Valley, PA, USA).

The cells were quantified as described before [E6]. Briefly, the quantification score was calculated by combining an estimate of the percentage of immunoreactivity of cells (quantity score) with an estimate of the staining intensity (staining intensity score). Percentage of immunoreactivity of cells was rated, on a scale of 0 to 4 as follows: no staining = 0; 1–10% of cells stained = 1; 11–50% of cells stained = 2; 51–80% of cells stained = 3; and 81–100% of cells stained = 4. Staining intensity was rated on a scale of 0 to 3, with 0 = negative; 1 = weak; 2 = moderate; and 3 = strong. The raw data were converted to the quantity data by
multiplying the quantity and staining intensity scores; with the scores ranging from 0 to 12.

**Shotgun proteomics**

**(1) CRSwNP samples and Protein Extraction**

In total, 30 subjects, including ten ECRSwNP patients, ten nonECRSwNP patients, and ten control subjects were recruited for shotgun proteomics. Details of subjects’ characteristics are shown in Table E1, and the cytokines assessed are shown in Figure E1 to reflect the inflammation bias for each group. For each sample, 100 mg of nasal polyps was ground into a powder in liquid nitrogen, homogenized on ice in 1 mL lysis buffer (7M urea, 2 M thiourea, 4 % CHAPS, 30 mM Tris-HCl, 1% protease inhibitor cocktail (Thermo Fisher Scientific), and sonicated (10× 10 s pulses) on ice. The homogenate was subjected to centrifugation (12,000 rpm) for 15 min at 4 °C. The supernatants from each individual were pooled as ECRSwNP, nonECRSwNP and control groups. Three volumes of ice-cold acetone were added to the pooled supernatants for protein precipitation at -20°C for 2 h. The solution was subjected to centrifugation (12,000 rpm) for 15 min at 4 °C, and then the precipitation was resolved in 5M urea. Protein concentrations were determined using the BCA protein assay kit (Beyotime, Nanjing, Jiangsu, China).

**(2) Trypsin Digestion**

100 µg of protein sample from each fraction was reduced with 10 mM DTT (Sigma-Aldrich, St Louis, MO, USA) at 37 °C for 2.5 h in the dark followed by alkylating with 50 mM iodoacetamide (Sigma-Aldrich) at room temperature for 40 min. Each sample was diluted with a solution of 50mM NH₄HCO₃ (Sigma-Aldrich), and the protein mixture was digested by modified sequencing-grade trypsin (Promega, Madison, WI, USA) at the ratio of 1:50 (enzyme: protein) at 37 °C for 20 h. The tryptic peptide mixture was lyophilized and stored at -80 °C until use.

**(3) LC-MS/MS**
The EttanMDLC system (GE Healthcare, Piscataway, NJ, USA) was applied for desalting and separation of tryptic peptide mixtures. Samples were desalted on RP trap columns (Zorbax 300 SB C18, Agilent Technologies, Palo Alto, CA, USA), and separated on an RP column (150 µm i.d., 100 mm length, Column Technology Inc., Fremont, CA, USA); using a mobile phase A (0.1% formic acid in HPLC-grade water) and the mobile phase B (0.1% formic acid in acetonitrile). Approximately 20 µg of each tryptic peptide mixture was loaded on to the columns and separated at a flow rate of 2 µL/min using a linear gradient of 4%-50% B for 120 min. The eluted peptides were detected using a FinniganLTQ linear ion trap MS (Thermo Electron, San Jose, CA, USA) equipped with an electrospray interface connected to the LC setup. Data-dependent MS/MS spectra were obtained simultaneously. Each scan cycle consisted of one full MS scan in profile mode followed by five MS/MS scans in centroid mode with the following dynamic exclusion settings: repeat count 2, repeat duration 30 s, exclusion duration 90 s.

(4) Data Analysis and Label-Free Quantitation

MS/MS spectra were automatically searched against the non-redundant Inter-national Protein Index (IPI) human protein database (version 3.68) using the Turbo SEQUEST program in the Bioworks Browser software suite (version 3.1, Thermo Electron). The peptides were constrained to be tryptic and up to two missed cleavages were allowed. Carbamidomethylation of cysteines was selected as a fixed modification, whereas oxidation of methionine residues was considered as variable modification. The mass tolerance allowed for the precursor and fragment ions was 3.0 Da and 1.0 Da, respectively. The stringent protein identification criteria were based on Delta Cn (≥0.1) and cross-correlation scores (Xcorr, one charge ≥1.9, two charges ≥2.2, three charges ≥3.75). Only proteins identified by at least two unique peptides were reported. Build Summary, an in-house tool, was used to combine the
peptide sequences into proteins and redundant proteins were deleted. To determine the false
discovery rate (FDR), the data set was searched against a sequence-reversed decoy IPI human
version 3.68 database using the same search parameters. FDR was calculated as follows:
\[ FDR = \frac{\text{Number of false peptides}}{\text{Number of true peptides} + \text{Number of false peptides}} \times 100\%. \]
Proteins were considered to be significantly changed between different samples only
when they met both criteria for \( P \)-value < 0.05 and the fold change >2.

**Subcellular localization and secreted proteins acquisition**

Proteins exclusively present in one group but absent in the other two groups, or expressed in
at least two groups and with the fold change higher than five were subjected to
COMPARTMENTS database [E7]. Proteins localized at “Exocytic vesicle”, “Exocytic
vesicle membrane”, “External side of plasma membrane”, “Extracellular exosome”,
“Extracellular exosome complex”, “Extracellular matrix”, “Extracellular matrix component”,
“Extracellular region”, “Extracellular region part”, “Extracellular space”, “Extracellular
space of host”, “Extracellular vesicle”, “Extrinsic component of cytoplasmic side of plasma
membrane”, “Extrinsic component of membrane”, and “Extrinsic component of plasma
membrane” were selected and then subjected to UniProt database (http://www.uniprot.org)
analysis to select the “secreted” proteins [E8].

**RNA isolation, reverse transcription, and real-time PCR**

Total RNA from sinus tissue was extracted with MiniBEST Universal RNA Extraction Kit
(TaKaRa Biotechnology, Dalian, Liaoning, China) according to the manufacturer’s
instructions. The quality of total RNA was assessed with Nanodrop-2000 (Thermo Fisher
Scientific).

Single-stranded cDNA was synthesized using PrimeScript™ RT Master Mix (TaKaRa
Biotechnology) and aliquots of cDNA equivalent to 10 ng of total RNA in each well were
used for real-time PCR.
Quantitative real-time PCR was performed with a SYBR Premix Ex Taq kit (TaKaRa Biotechnology) using an Applied Biosystems ViiA 7 Dx System (Applied Biosystems, Foster City, Calif, USA), in 10-µL reaction mixtures (5 µL of 2×SYBR Premix EX Taq, 0.2 µL ROX, 0.2 µmol/L of each primer; Table E3). Amplification was as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 sec, 60 °C for 1 min. After PCR, a melting curve was constructed by increasing the temperature from 60 to 95 °C with a temperature transition rate of 0.1°C/s. Relative gene expression was calculated as the $2^{-\Delta Ct}$ normalized to GAPDH, where $\Delta Ct= (Ct(\text{Target gene})- Ct(\text{GAPDH})$. No template sample was used as negative control.

**Elisa assay**

Freshly obtained tissue specimens were weighed, and 1 mL radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime) with 1% protease inhibitor cocktail (Thermo Fisher Scientific) was added for every 100 mg of tissue. The tissue samples were then homogenized using a standard bench-top homogenizer (Qiagen, Valencia, CA, USA) at 1000 rpm for 5 minutes, and the homogenates were centrifuged at 12000 rpm for 15 minutes at 4°C. The supernatants were aspirated and stored at −80°C until analyzed for Cystatin SN.

For isolated eosinophil cultures, the cells and media were harvested and centrifuged at 300×g for 10 min at 4°C. For epithelial cells, the media in the lower chamber of transwells were harvested. The cell-free supernatants or medium were stored at −80°C until analyzed for Cystatin SN, eosinophilic cationic protein (ECP) and eosinophil peroxidase (EPX).

ECP and EPX levels released from eosinophils in the supernatant were determined using specific Elisa kits obtained from Cusabio Life Sciences, Wuhan, China. The concentrations of Cystatin SN were determined according to the manufacturer’s instructions using a specific Elisa kit (Sigma-Aldrich). The color intensity of the Elisa reaction was measured with a Spectramax Plus reader (Molecular Devices, San Jose, CA, USA); and the
concentration of Cystatin SN, ECP or EPX in the cell lysate were normalized to the concentration of total protein, as detected with the BCA protein assay kit (Beyotime). The lower detection limits are listed in Table E4. Predict values were determined for those lower than the detection limits by the Spectramax Plus reader by standard curve automatic extension.

**Human nasal epithelial cell (HNEC) culture and treatment**

Nasal mucosa from control subjects and polyp tissues from patient with CRSwNP were digested with protease K in DMEM medium (0.05% w/v) at 4°C for 18 hours. At the end of this incubation 1/10 volume of FBS was added to stop the reaction and the solution was vortexed for 20 seconds, before centrifugation of the cell homogenates at 800 rpm for 5 minutes at 4°C. The supernatant was separated and the cells were resuspended and centrifuged further at 300xg for 10 minutes at 4°C to obtain the remaining cells. The cells were suspended in DMEM medium and incubated in 35 mm dish for one hour to remove residual fibroblasts. The medium was collected, centrifuged at 800 rpm for 5 minutes at 4°C, and the purified HNEC then re-suspended in bronchial epithelium basal medium (BEBM).

Cells were seeded in a 6.5-mm diameter polyester membrane with a pore size of 0.4 μm (Corning Costar, Corning, NY, USA) at a density of 1x10^5 cells in BEBM medium. The cells were cultured at 37°C, with 5% CO_2. When the density was up to 70%, the medium was changed to ALI medium (DMEM mixed 1:1 with BEBM) to develop ALI cultures. After the cells grew to complete confluence, the ALI medium in the upper layer of transwell was removed to allow the differentiation of cells. At the same time, transepithelial electrical resistance (TER) was measured daily in Ω x cm² by using a Millicell ERS Volt-Ohm Meter (Millipore, Temecula, Calif, USA), as described previously [E9]. When the TER reached a plateau, the ALI cultures were stimulated by adding cytokines to the lower compartment as follows: IL-3 100ng/mL (R&D systems, Minneapolis, MN, USA); IL-4 100ng/mL
(PeproTech, Rocky Hill, NJ, USA); IL-5 10ng/mL (R&D systems); CXCL8 100ng/mL (PeproTech); IL-10 100ng/mL (PeproTech); IL-13 100ng/mL (R&D systems); IL-17A 100ng/mL (PeproTech); IFN-γ 100ng/mL (R&D systems); GM-CSF 10ng/mL (R&D systems); cathepsin (CTS)B 100ng/mL (R&D systems); CTSC 100ng/mL (R&D systems).

Luminex

To explore the immunologic type of tissues subjected to the Shotgun proteomics, we evaluated the cytokine pattern in the local tissue. Samples were weighed and a total of 1.0 mL radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime) with 1% protease inhibitor cocktail (Thermo Fisher Scientific) was added per 100 mg of tissue. All samples were homogenized using a standard bench-top homogenizer (Qiagen) at 1000 rpm for 5 minutes and the homogenates were centrifuged at 12000 rpm for 15 minutes at 4°C. The supernatants were collected and stored at -80°C until further analysis for IL-5, CXCL8, IL-17A, GM-CSF, IFN-γ and TNF-α in tissue homogenates using ProcartaPlex™ Multiplex Immunoassay (Thermo Fisher Scientific) and analyzed on a Bio-Plex200 system (Biorad, Hercules, CA, USA).

For the dispersed nasal polyp cells (DPNC) and eosinophils treated with recombinant Cystatin SN or E64d, cell-free supernatants were collected and stored at -80°C until further analysis. The concentrations were normalized to total proteins, analyzed using BCA protein assay kit (Beyotime, Nanjing, Jiangsu, China). IL-1β, IL-4, IL-5, IL-10, IL-13, IL-17A, and IFN-γ from DNPC and eosinophils; and IL-5, CCL5, CCL11, CCL24, CCL26, and GM-CSF from eosinophils were determined by Fluorokine MAP Multiplex Kits (R&D systems, Minneapolis, MN, USA) and analyzed on a Bio-Plex200 system (Biorad). The lower detection limits are listed in Table E4.

In situ hybridization (ISH)

ISH for mRNA expression of CST1 was performed on polyp tissue sections. Tissues were
fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin. Tissue was sectioned at 6 µm using a Leica RM2235 Cryostat (Leica Microsystems). After deparaffinization and rehydration, sections were pretreated with proteinase K (5µg/mL) at 37°C for 10 min, and then washed with 0.1M glycine for 10 min. The sections were re-fixed in 1% paraformaldehyde for 20 min, and hybridized with DIG-labeled CST1 probes at 55°C for 16 hours, followed by washing with 2×SSC/50% formamide three times at 55°C. The signal was detected using an alkaline phosphatase-conjugated anti-DIG antibody (11093274910, Roche, Shanghai, China). Color development was achieved with 4-nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate, which rendered positive cells dark blue. Probes for CST1 were generated using DIG RNA Labeling Kit (11 175 025 910, Roche). Digoxigenin dUTP-labeled antisense riboprobes were generated from a 557-bp fragment subcloned from the N terminus of full-length human CST1 cDNA (position 83–639; accession no. NM_001898.2).

**Eosinophil isolation and culture**

Eosinophils were isolated using the Eosinophil Isolation Kit (MiltenyiBiotec, San Diego, CA, USA). Fresh peripheral blood from patients with ECRSwNP treated with heparin was mixed with the same volume of PBS containing 2mM EDTA. Then the cell suspension was mixed with the same volume of Ficoll-Paque (ρ = 1.077 g/mL) and centrifuged at 600×g for 30 minutes at 20 °C. The plasma and the mononuclear cells were gently aspirated away, and the undisturbed red cell pellet was re-suspended in 1× Red Blood Cell Lysis Solution, before incubating for 10 minutes at room temperature. The cell suspension was then centrifuged at 300×g for 8 minutes at 20 °C and the supernatant aspirated completely. The remaining cells pellet was washed with buffer and centrifuged at 300×g for 10 minutes and the resulting cell pellet was re-suspended in 40 µL of buffer and 10 µL of Biotin-Antibody Cocktail; which was incubated for 10 minutes at 4°C. At the end of this incubation 20 µL of Anti-Biotin
MicroBeads and 30 µL of buffer were added and the cell suspension incubated for an additional 15 minutes at 4°C. The cells were again washed with buffer and centrifuged at 300×g for 10 minutes. The supernatant was aspirated completely and the cell pellet was resuspended in the buffer before subjecting to magnetic separation. The unlabeled cells and total effluent pass through the separating column were collected and the column was washed with the appropriate amount of buffer. The cells were centrifuged at 300×g for 10 min at 4°C, resuspended in cell culture media, and then cultured in 96-well plate in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), at a concentration of 10^5 cells per well. The purification of the isolated eosinophils was detected by flow cytometry (Figure E5). For each treatment, at least three parallel wells were established and 100ng/mL recombinant Cystatin SN (R&D systems) or 10 µM E64d (Cayman Chemical, Michigan, USA) were used to treat the cells for 24 to 48 hours. For several samples, 100ng/mL recombinant IL-5 (R&D systems) or 100ng/mL recombinant GM-CSF (R&D systems) were used to treat the cells for 24 hours as positive control. At the end of the incubation period, the cells and the cell-free media were harvested for further analysis.

**Flow cytometry**

For CST1 expression in peripheral blood, white blood cells acquired from fresh blood cells at baseline or cells treated with IL-4 for 24 hours were analyzed. Cells were lysed with FACSTM Lysing solution (BD PharMingen, Franklin Lakes, NJ, USA), and the remaining cells were permeabilized using FACSTM permeabilizing solution (BD PharMingen). Following washing with buffer, the cells were stained with APC-conjugated anti-CST1 antibody at room temperature in the dark for 30 min. At the end of this incubation, the cells were washed again and re-suspended in 1% paraformaldehyde for analysis.

For eosinophil purification test, isolated eosinophils were stained with Alexa Fluor 700-
conjugated Siglec-8 antibody for 30 min at 4°C in the dark; For eosinophil activation assay, isolated eosinophils were stained with FITC-conjugated CD69 antibody for 30 min at 4°C in the dark. The stained cells were analyzed using Attune NxT flow cytometry (Thermo Electron, San Jose, CA). The detailed information of primary antibodies used was listed in Table E2.

**Dispensed nasal polyp cells (DNPC) culture**

Nasal polyp tissues were digested using 2 mg/mL type II collagenase (Worthington, Lakewood, NJ, USA) and 0.04 mg/mL deoxyribonuclease I (DNase I) (Sigma-Aldrich) in RPMI 1640 medium (Thermo Fisher Scientific), at 37 °C for 2 hours. At the end of the incubation, the tissues were dissociated further by mechanical dissociation using the Gentle MACS Dissociator (MiltenyiBiotec, San Diego, CA, USA), and the resulting cell suspension was filtered through a 70 µm cell strainer (BD Biosciences, San Jose, CA, USA). The filtered cells were centrifuged at 300×g for 10 min and the cell pellets were re-suspended in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS)(Thermo Fisher Scientific).

**Eosinophils migration assay**

Migration assays were performed using transwell plates with 5 µm pore inserts (Costar, Cambridge, MA, USA), as recommended by the manufacturer. Eosinophils isolated from peripheral blood were grown in the upper layer of the transwell at a concentration of 5×10^5 cells in 200 µL of RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS)(Thermo Fisher Scientific). The lower compartments of the transwells were contained the same medium with 100 ng/mL recombinant Cystatin SN or 10 µM E64d. For each treatment, three wells were established at each time point for observation: at one, two, three and four hours, respectively. The medium in the lower compartments was collected at each time point and then centrifuged at 300×g for 10 min. The cell pellets were
re-suspended in RPMI 1640 medium and then assessed for the number of eosinophils migrating through the transwell by counting with a hemocytometer.

**Eosinophils recruitment**

Polyp tissues were rinsed in PBS containing 1% penicillin, 1% streptomycin, and 1% amphotericin, and then weighed before cutting into smaller sections about 0.1g in weight. Separate sections were treated by injecting with 10 µL of 10 µg/mL recombinant Cystatin SN or 1 mM E64d or PBS, as well as 10 µL of PBS or 10 µg/mL IL-5 blocking antibody (Abcam, Cambridge, UK), according to the model shown in Figure E9. The treated sections were then co-cultured with white blood cells (WBCs) for 96 hours, and at the end of this incubation, the sections were collected and embedded in paraffin for H&E staining. Several polyps sections were injected with 10 µL of 10 µg/mL recombinant IL-5, 10 µg/mL recombinant GM-CSF, or 10 µg/mL recombinant CCL24 as positive control. The WBCs were prepared as shown below and used at a concentration of $10^6/600\mu$L in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) for each well.

WBCs were separated from heparinized peripheral blood from patients with CRSwNP. The blood from each patient was centrifuged at 300×g for 10 min at 4 °C and the plasma was discarded. The cell pellet was re-suspended with the seven-fold volume of Red Blood Cell Lysis Solution (TBDscience, Tianjin, China) and incubated for 2 min at 4°C before centrifugation again at 300×g for 10 minutes at 4°C. The supernatant was carefully aspirated completely and the cell pellet was resuspended in PBS for further centrifugation at 300×g for 10 min to obtain the final washed pellet.
REFERENCES


### Table E1: Clinical features of patients.

<table>
<thead>
<tr>
<th>Methodology used</th>
<th>Control</th>
<th>ECRSwNP</th>
<th>nonECRSwNP</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total subjects enrolled</strong></td>
<td>40</td>
<td>192</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td><strong>Subject number</strong>:</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Gender, female</strong>:</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td>0.670</td>
</tr>
<tr>
<td><strong>Age (years±SD)</strong>:</td>
<td>39±18</td>
<td>48±12</td>
<td>45±12</td>
<td>0.318</td>
</tr>
<tr>
<td><strong>Patients with atopy</strong>:</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0.286</td>
</tr>
<tr>
<td><strong>Patients with AR</strong>:</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Patients with asthma</strong>:</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Real time-PCR and Elisa for CST1 expression</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Subject number</strong>:</td>
<td>20</td>
<td>44</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td><strong>Gender, female</strong>:</td>
<td>10</td>
<td>20</td>
<td>7</td>
<td>0.443</td>
</tr>
<tr>
<td><strong>Age (years±SD)</strong>:</td>
<td>42±15</td>
<td>46±14</td>
<td>49±13</td>
<td>0.420</td>
</tr>
<tr>
<td><strong>Patients with atopy</strong>:</td>
<td>5</td>
<td>20</td>
<td>4</td>
<td>0.056</td>
</tr>
<tr>
<td><strong>Patients with AR</strong>:</td>
<td>0</td>
<td>16</td>
<td>0</td>
<td>0.000*</td>
</tr>
<tr>
<td><strong>Patients with asthma</strong>:</td>
<td>0</td>
<td>19</td>
<td>0</td>
<td>0.000*</td>
</tr>
<tr>
<td><strong>IHC and ISH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Subject number</strong>:</td>
<td>5</td>
<td>8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><strong>Gender, female</strong>:</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>0.712</td>
</tr>
<tr>
<td><strong>Age (years±SD)</strong>:</td>
<td>34±12</td>
<td>48±12</td>
<td>39±15</td>
<td>0.333</td>
</tr>
<tr>
<td><strong>Patients with atopy</strong>:</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Patients with AR</strong>:</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Patients with asthma</strong>:</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0.477</td>
</tr>
<tr>
<td><strong>Flow cytometry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Subject number</strong>:</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td><strong>Gender, female</strong>:</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Age (years±SD)</strong>:</td>
<td>-</td>
<td>41±13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Patients with atopy</strong>:</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Patients with AR</strong>:</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Patients with asthma</strong>:</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Cell culture</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Subject number</strong>:</td>
<td>5</td>
<td>114</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Gender, female</strong>:</td>
<td>2</td>
<td>53</td>
<td>6</td>
<td>0.676</td>
</tr>
<tr>
<td><strong>Age (years±SD)</strong>:</td>
<td>41±10</td>
<td>46±11</td>
<td>46±17</td>
<td>0.751</td>
</tr>
<tr>
<td>Patients with atopy</td>
<td>0</td>
<td>25</td>
<td>1</td>
<td>0.345</td>
</tr>
<tr>
<td>---------------------</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>-------</td>
</tr>
<tr>
<td>Patients with AR</td>
<td>0</td>
<td>21</td>
<td>1</td>
<td>0.465</td>
</tr>
<tr>
<td>Patients with asthma</td>
<td>0</td>
<td>29</td>
<td>0</td>
<td>0.085</td>
</tr>
</tbody>
</table>

### Eosinophils recruitment in NP

<table>
<thead>
<tr>
<th>Subject number</th>
<th>0</th>
<th>5</th>
<th>5</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, female</td>
<td>-</td>
<td>3</td>
<td>2</td>
<td>1.000</td>
</tr>
<tr>
<td>Age (years±SD)</td>
<td>-</td>
<td>40±8</td>
<td>51±8</td>
<td>0.056</td>
</tr>
<tr>
<td>Patients with atopy</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>Patients with AR</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>Patients with asthma</td>
<td>-</td>
<td>1</td>
<td>0</td>
<td>1.000</td>
</tr>
</tbody>
</table>
Table E2. Antibodies used in immunohistochemistry and flow cytometry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Application</th>
<th>Species</th>
<th>Concentration</th>
<th>Clone ID</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD69 (FITC - conjugated)</td>
<td>FC</td>
<td>Mouse</td>
<td>1:20</td>
<td>FN50</td>
<td>Biolegend</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human Cystatin-SN (CST1) (APC -</td>
<td>FC</td>
<td>Rabbit</td>
<td>1:100</td>
<td>32097-05161</td>
<td>AssayPro</td>
</tr>
<tr>
<td>conjugated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Siglec-8(Alexa Fluor 700 -</td>
<td>FC</td>
<td>Mouse</td>
<td>1:20</td>
<td>#837535</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>conjugated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-cystatin SN antibody</td>
<td>IHC</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Ab124281</td>
<td>Abcam</td>
</tr>
<tr>
<td>HRP/Fab Polymer -conjugated</td>
<td>IHC</td>
<td>Rabbit</td>
<td>Working solution</td>
<td>PV6001</td>
<td>ZSGB-BIO</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table E3. Primers used for real-time PCR analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CST1</td>
<td>5'-CGGGTGCCATCTATAACGCA-3'</td>
<td>5'-GTCTGTTGCCTGGCTCTTAGT-3'</td>
<td>139</td>
</tr>
<tr>
<td>EPX</td>
<td>5'-GTCCTCGGAGACTGCATAGC-3'</td>
<td>5'-TATAATCTGCGGCCCGAACA-3'</td>
<td>175</td>
</tr>
<tr>
<td>ECP</td>
<td>5'-CCCACAGTTTACGAGGGCTC-3'</td>
<td>5'-ACCCGGAATCTACTCCGATGA-3'</td>
<td>228</td>
</tr>
<tr>
<td>PU.1</td>
<td>5'-GCGACCATTACTGGGACTTCC-3'</td>
<td>5'-GGGTATCGAGGACGTGCAT-3'</td>
<td>156</td>
</tr>
</tbody>
</table>
Table E4. The lower limit of detection for Elisa and Luminex kits

<table>
<thead>
<tr>
<th>Target</th>
<th>Lower limit</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-5</td>
<td>7.42 pg/mL</td>
<td>Luminex (ProcartaPlex™)</td>
</tr>
<tr>
<td>CXCL8</td>
<td>2.4 pg/mL</td>
<td>Luminex (ProcartaPlex™)</td>
</tr>
<tr>
<td>IL-17A</td>
<td>7.2 pg/mL</td>
<td>Luminex (ProcartaPlex™)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>10.01 pg/mL</td>
<td>Luminex (ProcartaPlex™)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>12.38 pg/mL</td>
<td>Luminex (ProcartaPlex™)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.6 pg/mL</td>
<td>Luminex (ProcartaPlex™)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5.43 pg/mL</td>
<td>Luminex (Fluorokine MAP) (Kit 1)</td>
</tr>
<tr>
<td>IL-4</td>
<td>5.14 pg/mL</td>
<td>Luminex (Fluorokine MAP) (Kit 1)</td>
</tr>
<tr>
<td>IL-5</td>
<td>2.09 pg/mL</td>
<td>Luminex (Fluorokine MAP) (Kit 1)</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.21 pg/mL</td>
<td>Luminex (Fluorokine MAP) (Kit 1)</td>
</tr>
<tr>
<td>IL-17A</td>
<td>3.28 pg/mL</td>
<td>Luminex (Fluorokine MAP) (Kit 1)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>16.39 pg/mL</td>
<td>Luminex (Fluorokine MAP) (Kit 1)</td>
</tr>
<tr>
<td>CCL5</td>
<td>7.76 pg/mL</td>
<td>Luminex (Fluorokine MAP) (Kit 2)</td>
</tr>
<tr>
<td>CCL11</td>
<td>20.23 pg/mL</td>
<td>Luminex (Fluorokine MAP) (Kit 2)</td>
</tr>
<tr>
<td>CCL24</td>
<td>6.39 pg/mL</td>
<td>Luminex (Fluorokine MAP) (Kit 2)</td>
</tr>
<tr>
<td>CCL26</td>
<td>4.77 pg/mL</td>
<td>Luminex (Fluorokine MAP) (Kit 2)</td>
</tr>
<tr>
<td>IL-5</td>
<td>2.21 pg/mL</td>
<td>Luminex (Fluorokine MAP) (Kit 2)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>4.07 pg/mL</td>
<td>Luminex (Fluorokine MAP) (Kit 2)</td>
</tr>
<tr>
<td>ECP</td>
<td>1.56 ng/mL</td>
<td>Elisa</td>
</tr>
<tr>
<td>EPX</td>
<td>3.12 ng/mL</td>
<td>Elisa</td>
</tr>
<tr>
<td>CST1</td>
<td>0.2 ng/mL</td>
<td>Elisa</td>
</tr>
</tbody>
</table>
Table E5. The expression of cathepsins (CTS) by data mining

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ECRSwNP /CTRL</td>
<td>ECRSwNP /CTRL</td>
<td>CRSwNP /CTRL</td>
<td>ECRSwNP /CTRL</td>
<td>mouse</td>
</tr>
<tr>
<td></td>
<td>nonECRSwNP /CTRL</td>
<td>nonECRSwNP /CTRL</td>
<td></td>
<td>nonECRSwNP /CTRL</td>
<td>eosinophils</td>
</tr>
<tr>
<td>CTSB</td>
<td>3.33</td>
<td>1.59</td>
<td>2.22</td>
<td>√</td>
<td>[E10, 11]</td>
</tr>
<tr>
<td>CTSC</td>
<td>5.66</td>
<td>2.33</td>
<td>3.05</td>
<td>√</td>
<td>[E10-12]</td>
</tr>
<tr>
<td>CTSF</td>
<td>0.57</td>
<td>0.47</td>
<td></td>
<td></td>
<td>[E10]</td>
</tr>
<tr>
<td>CTSK</td>
<td>0.36</td>
<td></td>
<td></td>
<td></td>
<td>[E10]</td>
</tr>
<tr>
<td>CTSL</td>
<td>2.01</td>
<td></td>
<td></td>
<td>√</td>
<td>[E10, 11]</td>
</tr>
<tr>
<td>CTSO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTSS</td>
<td></td>
<td></td>
<td></td>
<td>√</td>
<td>[E10, 11]</td>
</tr>
<tr>
<td>CTSV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTSW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTSZ</td>
<td>1.01</td>
<td>1.80</td>
<td></td>
<td>√</td>
<td>[E11]</td>
</tr>
</tbody>
</table>

ECRSwNP: eosinophilic CRSwNP; nonECRSwNP: non-eosinophilic CRSwNP; CTRL: control; CTS: cathepsins
FIGURE LEGENDS

FIGURE E1. Measurement of IL-5, IL-17A, IFN-γ, GM-CSF, CXCL8 and TNF-α using Luminex assay for homogenates of same tissues applied for proteomics.

FIGURE E2. CST1 expression in hematopoietic cells at baseline (Upper line) and in cells treated with 100ng/mL IL-4 for 24 hours (Lower line).

FIGURE E3. Effect of inflammatory mediators on the expression of CST1 in nasal epithelial cells. CST1 mRNA was measured in epithelial cells from nasal mucosa from control subjects and polyp tissues, following incubation with cathepsin B (CTSB), CTSC, IL-3, IL-5, GM-CSF, IFN-γ, CXCL8 (IL-8), and IL-10.

FIGURE E4. CST1 activates eosinophils. The relative mRNA expression levels of ECP and EPX for dispersed nasal polyp cells (DPNC). DNPC were treated with medium (mock), 100ng/mL recombinant Cystatin SN or 10µM E64d for 24 hours (n=15). For each gene, samples from the same individual are shown in the same color, and the mean values for each group are shown as the green squares.

FIGURE E5. The purity of the eosinophil blood preparations using eosinophil isolation kit detected by flow cytometry.

FIGURE E6. The effect of CST1, IL-5, GM-CSF on the activation of eosinophils isolated from peripheral blood of patients with ECRSwNP. Isolated eosinophil cultures were treated with 100ng/mL recombinant Cystatin SN, 100ng/mL recombinant IL-5, or 100ng/mL recombinant GM-CSF for 24 hours and then assessed for A, the relative expression of ECP, EPX and PU.1 mRNAs in eosinophils; B, concentrations of ECP and EPX released in the culture medium; C, CD69 expression in eosinophils. For each gene or protein, samples from the same individual are shown in the same color, and the mean values for each group are shown as the green squares.

FIGURE E7. Eosinophil migration induced by recombinant Cystatin SN and E64d (n=6).
FIGURE E8. CST1, IL-5, GM-CSF, Eotaxin-2 promotes recruitment of eosinophils. A, nasal polyp sections were injected with 10 µg/mL recombinant Cystatin SN, 10 µg/mL recombinant IL-5, 10 µg/mL recombinant GM-CSF, 10 µg/mL recombinant Eotaxin-2 or 1 mM E64d in the volume of 10 µL per 0.1 g tissue and then incubated with white blood cells isolated from the individual from whom the polyp tissue was obtained. After incubation for 96 h, the polyp sections were collected and following H&E staining were assessed for the number of eosinophils present. Bars = 50 µm (200× magnification); B, average numbers of eosinophils recruited in each group of five scopes under 400× magnification (high power field, HPF). (n=7 in total).

FIGURE E9. Schematic diagram showing the injection model for eosinophil recruitment in nasal polyp tissue. For patients with CRSwNP, polyps and peripheral blood were obtained simultaneously during surgery. White blood cells (WBCs) at a concentration of 10^6/600 µL RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) were added to each well of 24-well plate. Polyp sections weighing approximately 0.1 g per section were injected with 10 µL of each recombinant protein, PBS and/or IL-5 blocking antibody, and co-cultured with WBCs for 96 hours.

FIGURE E10. Effect of recombinant Cystatin SN on the expression of eosinophil chemo-attractant cytokines released from eosinophils. Cultured eosinophils were treated with 100 ng/mL recombinant Cystatin SN or PBS for 24 hours, and the cell-free supernatants were collected for assessment of cytokines by Luminex (n=8). *: P < 0.05.

FIGURE E11. Schematic diagram showing the regulation and function of CST1 in CRSwNP. The IL-4 and IL-13-induced epithelial Cystatin SN enhances the activation and recruitment of eosinophils via IL-5, which acts in an autocrine manner in the eosinophils to form a feedback loop to expand the Th2-mediated inflammation. In the presence of large numbers of
neutrophils, IL-17A generated by the neutrophils or Th17 cells inhibits the expression of Cystatin SN, and thus attenuates the progression of eosinophilic inflammation via this route.