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Epithelial Lining Fluid Cystatin SN is a Noninvasive Biomarker for Predicting Type 2 Chronic Rhinosinusitis

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Keywords: biomarker | chronic rhinosinusitis | cystatin SN | endotype | type 2 inflammation

ABSTRACT

Background: As treatment responses differ according to the inflammatory endotype of chronic rhinosinusitis (CRS), identifying the endotype could facilitate personalized treatment. We aimed to identify a noninvasive epithelial lining fluid (ELF) biomarker for the type 2 (T2) endotype of CRS.

Methods: Nasal tissue and ELF samples were obtained from patients with CRS and control individuals. Single-cell RNA sequencing (scRNA-seq) data were analyzed. The expression of inflammatory mediators was measured using Luminex multiplex assays and enzyme-linked immunosorbent assays.

Results: Analysis of the scRNA-seq data revealed that *CST1* was exclusively expressed in epithelial cells of T2 CRS, but not in those of non-T2 CRS, and this was confirmed by immunofluorescence staining. Cystatin SN expression was detected in ELF, and its levels were significantly higher in T2 CRS than in non-T2 CRS and controls. The expression level of cystatin SN in the ELF was positively correlated with the Lund-Mackay CT, SNOT-22, and JESREC scores, whereas it was inversely correlated with olfactory function. Furthermore, ELF cystatin SN levels correlated with the tissue/blood eosinophil count and nasal tissue expression of T2 inflammatory mediators, indicating that ELF cystatin SN is a reliable marker of nasal T2 inflammation. In the receiver operating characteristics curve analysis evaluating the predictive efficacy for T2 CRS, the AUC for ELF cystatin SN was 0.894 (0.936 in the validation cohort), which was higher than that of other markers, including blood eosinophil count, serum total IgE level, and the JESREC score. With a cut-off value of 112.5 ng/mg, ELF cystatin SN yielded a 75.0% sensitivity and 92.0% specificity.

Conclusions: ELF cystatin SN is a clinically feasible, noninvasive biomarker with superior accuracy for predicting T2 CRS.

Abbreviations: AUC, area under the curve; CRS, chronic rhinosinusitis; CST1, cystatin SN; DEG, differentially expressed genes; ELF, epithelial lining fluid; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; JESREC score, Japanese epidemiological survey of refractory eosinophilic chronic rhinosinusitis; scRNA-seq, single-cell RNA sequencing; SNOT-22, 22-item Sino-Nasal Outcome Test.

Seojin Moon and Sungmin Moon contributed equally to this work.

1 | Introduction

Chronic rhinosinusitis (CRS) is a chronic inflammatory disease of the nasal cavity and paranasal sinuses with a high prevalence worldwide [1], causing a substantial healthcare burden. CRS is traditionally classified as CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP) [2, 3]. CRSwNP represents a particularly challenging subtype [4–6], characterized by severe disease symptoms and high rates of recurrence [7]. Recently, endotyping based on the inflammatory status of the mucosa has been implemented to better understand the heterogeneity of CRS and to develop tailored treatment strategies [8, 9].

CRS has been divided into type 2 (T2) and non-T2 endotypes, based on the degree of T2 inflammation [10]. T2 CRS is characterized by predominant eosinophilic infiltration into the tissue and elevated expression of T2 cytokines, including interleukin (IL)-4, IL-5, and IL-13, whereas non-T2 CRS comprises subendotypes with type 1 and/or type 3 inflammation [11–13]. Accumulating evidence has shown that clinical presentations and treatment responses differ according to inflammatory endotype [14]. Therefore, identifying the inflammatory endotype before treatment initiation can inform personalized treatment options for patients with CRS. As biologics targeting T2 inflammation have shown great efficacy in real-world settings [15], the identification of suitable patients is critical. Given that only 25%–63% of patients with CRSwNP exhibit T2 inflammatory profiles in East Asia [16–18], accurate methods for distinguishing T2 CRS are particularly needed in these countries.

Currently, a definitive diagnosis of T2 CRS is made by determining eosinophil infiltration in the sinonasal tissue [19–21]. This requires invasive procedures such as biopsy and surgery, and time-consuming histopathological evaluation. Several noninvasive markers have been suggested to identify the presence of T2 inflammation [22, 23]. Recently, the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS)/European Forum for Research and Education in Allergy and Airway Diseases (EUFOREA) guidelines suggested peripheral eosinophilia (blood eosinophil count $\geq$150/μL) and elevated serum total immunoglobulin E (IgE) levels (IgE $\geq$100 IU/mL) as evidence of T2 inflammation [3, 24]. However, the cut-off values of these parameters lacked a robust scientific background. Therefore, there is a growing need for noninvasive, rapid, and accurate methods to diagnose T2 CRS. Because nasal epithelial lining fluid (ELF) collection induces minimal discomfort in patients compared to tissue biopsy or other procedures used for the lower airways, a biomarker using this sampling technique would be clinically feasible and could guide personalized treatment for patients with T2 CRS and provide a new objective measure for predicting disease progression and treatment.

In the present study, we aimed to identify a noninvasive ELF biomarker for predicting T2 CRS. In the analysis of publicly available transcriptomic data, we identified epithelial-derived cystatin SN as a T2-specific biomarker candidate. We found that cystatin SN expression in ELF was significantly higher in T2 CRS compared to non-T2 CRS. In addition, cystatin SN levels in ELF significantly correlated with clinical disease severity and T2 inflammation. We also assessed the efficacy of ELF cystatin SN as a biomarker by analyzing its accuracy in distinguishing T2 CRS from non-T2 CRS.

2 | Methods

2.1 | Study Subjects

A total of 53 patients with CRS who underwent endoscopic sinus surgery (ESS) at Severance Hospital, Seoul, Republic of Korea, from April 2022 to June 2024, were enrolled in this study (Cohort 1; discovery cohort). CRS diagnosis was based on the EPOS 2020 guidelines [3]. The inclusion criteria for study subjects were as follows: (1) age $\geq$18 years and diagnosed with CRS, (2) had undergone ESS, and (3) agreed to participate in the study. Patients diagnosed with fungal sinusitis, cystic fibrosis, or other autoimmune diseases were excluded, as were those who had been treated with oral/topical corticosteroids within 4 weeks before surgery or those treated with biologics/immunosuppressive agents. Nasal tissue and/or ELF samples were obtained from the patients. In tissue samples stained with hematoxylin and eosin, eosinophil counts were quantified in five high-power fields at ×400 magnification. According to previous studies [25, 26], the patients were categorized into two groups: T2 CRS (eosinophils >10% of the inflammatory cells; $n=28$) and non-T2 CRS (eosinophils $\leq$10% of the inflammatory cells; $n=25$). Clinical information, including age, sex, body mass index (BMI), asthma comorbidity, atopic status, and laboratory test results, was obtained. The atopic status was determined using multiple allergen simultaneous tests (AdvanSure AlloScreen; LG Life Sciences, Korea) [27] and/or skin prick tests. Computed tomography (CT) scans were performed before surgery, and the Lund-Mackay CT score was obtained to evaluate disease severity [28]. Olfactory function was evaluated using the YSK olfactory function test (YOF test; Kimex Co., Suwon, Korea) [29] and visual analog scale (VAS) scores. VAS scores ranged from 0 to 10, with higher scores indicating poorer olfactory function. The patients also completed the 22-item Sino-Nasal Outcomes Test (SNOT-22), a disease-specific quality-of-life metric [30]. Additionally, we obtained ethmoid mucosal tissue samples or ELFs from 14 control individuals without evidence of CRS during other rhinologic surgeries such as septoplasty, skull base surgery, or removal of mucous retention cysts in the maxillary/sphenoid sinus. The details of patients' characteristics are presented in Table 1. Additionally, a validation cohort comprising 50 patients with CRS and 16 control individuals (Cohort 2) was prospectively enrolled at Severance Hospital between September 2024 and March 2025 (Table 2). This study was reviewed and approved by the institutional review boards of Severance Hospital (No. 4-2021-0573). Informed consent was obtained from all participants, and the study was conducted in accordance with the principles of the Declaration of Helsinki.

2.2 | Epithelial Lining Fluid Collection

ELF was collected from each patient's nasal cavity using Leukosorb strips [31]. The strips were inserted into the middle meatus, removed after 5 min, and placed in a 1.5 mL microcentrifuge tube (Supplementary Video). The strips were then moved to 650 μL microcentrifuge tubes, which were modified to have a hole at the tip. Tubes containing strips were placed in a 1.5 mL microcentrifuge tube containing 150 μL of Dulbecco's phosphate-buffered saline with 1% bovine serum albumin (BSA) and 0.05% Triton X-100 (GIBCO/Thermo Fischer, Waltham,

TABLE 1 | Demographic and clinical characteristics of Cohort 1 (discovery cohort).

Parameter	Control ($n=14$)	Patients with CRS ($n=53$)		p
		Non-T2 ($n=25$)	T2 ($n=28$)	
Sex (male/female)				0.3370
Male, *n* (%)	11 (78.6)	21 (84.0)	20 (71.4)	
Female, *n* (%)	3 (21.4)	4 (16.0)	8 (28.6)	
Age, mean ± SD	42.9 ± 16.9	47.8 ± 19.7	51.2 ± 16.8	0.6050
BMI, mean ± SD	24.5 ± 3.7	25.0 ± 2.8	24.8 ± 2.9	0.1864
Nasal polyp status				0.5966
Present, *n* (%)	N/A	23 (92.0)	27 (96.5)	
Absent, *n* (%)	N/A	2 (8.0)	1 (3.5)	
Asthma, *n* (%)	0 (0)	2 (8.0)	6 (21.4)	0.2564
Atopy (allergic sensitization), *n* (%)	7 (50)	16 (64.0)	18 (64.2)	0.9827
Lund–Mackay CT score	N/A	13.0 ± 5.8	16.0 ± 5.7	**0.0496**
SNOT-22 score, mean ± SD	N/A	33.9 ± 19.8	41.0 ± 24.2	0.3640
JESREC score, mean ± SD	N/A	9.8 ± 3.5	12.4 ± 3.1	**0.0054**
Revision status (yes/no)				0.5091
Yes, *n* (%)	N/A	4 (16.0)	7 (25.0)	
No, *n* (%)	N/A	21 (84.0)	21 (75.0)	
Olfaction				
TDI score, mean ± SD	N/A	18.4 ± 7.1	16.1 ± 6.5	0.3186
VAS score, mean ± SD	N/A	6.0 ± 2.8	7.4 ± 2.8	0.0838

Abbreviations: BMI, body mass index; CRS, chronic rhinosinusitis; CT, computed tomography; JESREC, Japanese epidemiological survey of refractory eosinophilic chronic rhinosinusitis; N/A, not applicable; SNOT-22, 22-item sino-nasal outcome test; VAS, visual analogue scale.

MA, USA). Strips were centrifuged for 5 min at 13,000 rpm to elute the ELF, and the supernatants without debris were stored at −80°C before assay.

2.3 | Tissue Homogenate Preparation

Nasal tissue was obtained during ESS. The tissue (0.1 g) was diluted in 1 mL 0.9% NaCl solution containing a protease inhibitor cocktail 1× (Roche Diagnostics, Mannheim, Germany). This mixture was homogenized using the TissueLyzer II (Qiagen, Hilden, Germany), and then the suspension was centrifuged at 4°C. The supernatant was separated and stored at −80°C.

2.4 | Immunofluorescence Staining

Nasal tissue was fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.1% Triton X-100 (0694; Amresco, Solon, Ohio, USA) for 5 min, and blocked with 5% BSA for 1 h. The cells were then incubated with primary antibodies and/or isotype control antibodies overnight at 4°C, followed by incubation with fluorescently labeled secondary antibodies for 1 h at room temperature. Nuclei were counterstained with DAPI. Confocal images were acquired using a confocal laser-scanning microscope (LSM980; Carl Zeiss Microscopy, München, Germany) and analyzed using ZEN image software (Carl Zeiss, ZEN 3.0 lite). The primary antibodies used for immunofluorescence staining were rabbit anti-cystatin SN (ab124281; Abcam, Cambridge, MA, USA), rabbit IgG polyclonal-isotype control (ab37415; Abcam), and mouse anti-EpCAM (ab252530; Abcam). The secondary antibodies were Alexa Fluor 488 donkey anti-rabbit IgG (A-21206; Thermo Fischer Scientific) and Alexa Fluor 568 goat anti-mouse IgG (A-11004; Thermo Fischer Scientific).

2.5 | Human Nasal Epithelial Cell (HNEC) Culture

HNECs were cultured as previously described [32]. Briefly, NP tissues from patients with CRSwNP were digested with proteinase K overnight at 4°C. Next, these tissues were transferred to bronchial epithelial growth medium (BEGM; CC-3170; Lonza, Walkersville, MD, USA) supplemented with the BEGM SingleQuots Kit (CC-4175; Lonza). The tissues were then scraped and centrifuged for 3 min at 1200 rpm at room temperature. Purified nasal epithelial cells were resuspended and cultured in a humidified incubator at 37°C and 5% CO_2. Next, the cells were detached using ReagentPack (CC-5034; Lonza), and 1×10^5 cells were seeded on transwell inserts (12 mm, 0.4-μm pore; 3460; Corning, NY, USA) using air–liquid interface (ALI) medium

TABLE 2 | Demographic and clinical characteristics of Cohort 2 (validation cohort).

Parameter	Control ($n=16$)	Patients with CRS ($n=50$)		p
		Non-T2 ($n=30$)	T2 ($n=20$)	
Sex (Male/Female)				0.5669
Male, *n* (%)	7 (43.7)	18 (60.0)	10 (50)	
Female, *n* (%)	9 (56.3)	12 (40.0)	10 (50)	
Age, mean ± SD	55.4 ± 17.8	50.0 ± 17.4	48.4 ± 23.8	0.7160
BMI, mean ± SD	22.2 ± 2.4	25.4 ± 3.8	23.8 ± 3.9	0.1507
Nasal polyp status				**0.0068**
Present, *n* (%)	N/A	21 (70.0)	20 (100)	
Absent, *n* (%)	N/A	9 (30.0)	0 (0.0)	
Asthma, *n* (%)	1 (6.3)	0 (0.0)	5 (25.0)	**0.0067**
Atopy (allergic sensitization), *n* (%)	6 (37.5)	9 (30.0)	15 (75.0)	**0.0017**
Lund–Mackay CT score	N/A	7.3 ± 5.9	14.6 ± 5.8	**< 0.001**
SNOT-22 score, mean ± SD	N/A	29.2 ± 21.5	35.8 ± 21.5	0.2801
JESREC score, mean ± SD	N/A	6.3 ± 3.1	11.9 ± 3.5	**< 0.001**
Revision status (yes/no)				0.3100
Yes, *n* (%)	N/A	8 (26.6)	3 (15.0)	
No, *n* (%)	N/A	22 (73.4)	17 (85.0)	
Olfaction				
TDI score, mean ± SD	N/A	20.8 ± 6.1	17.6 ± 3.9	0.1025
VAS score, mean ± SD	N/A	4.2 ± 3.2	6.1 ± 2.8	0.0563

Abbreviations: BMI, body mass index; CRS, chronic rhinosinusitis; CT, computed tomography; JESREC, Japanese epidemiological survey of refractory eosinophilic chronic rhinosinusitis; N/A, not applicable; SNOT-22, 22-item sino-nasal outcome test; VAS, visual analogue scale.

prepared by mixing BEGM 1:1 with DMEM (11995–065; Gibco, NY, USA). Upon reaching confluence, the apical compartments were exposed to air to allow cell differentiation. The medium was replaced every other day for 14 days. For the cystatin SN secretion assay, HNECs were stimulated by the addition of recombinant human IL-4 (200–04; PeproTech, Rocky Hill, NJ, USA) to the basolateral medium at a concentration of 100 ng/mL for 7 days. The IL-4-containing medium was replaced every other day. On Days 1, 3, 5, and 7 after IL-4 treatment, the apical supernatant was collected by washing with PBS.

2.6 | Measurement of Protein Expression

The concentrations of cystatin SN in both tissue homogenates and ELFs were measured using a human cystatin SN enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. The concentrations of cytokines in nasal tissue homogenates, including IFN-γ, IL-5, IL-17A, CCL24/eotaxin-2, and CCL26/eotaxin-3, were analyzed using the Human Luminex Discovery Assay (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. All protein levels in the ELF or tissue homogenates were normalized to the concentration of total protein [33, 34].

2.7 | Analysis of Public RNA-Seq Data

From a publicly available scRNA-seq dataset (Genome Sequence Archive HRA000772) [35], we obtained data from control individuals (HRS162345, HRS162346, HRS162347, HRS162348, and HRS162349) and patients with CRSwNP (HRS162329, HRS162330, HRS162331, HRS162332, HRS162333, HRS162334, HRS162335, HRS162336, HRS162337, HRS162338, and HRS162339). A raw gene expression matrix was generated using Cell Ranger version 7.1.0 (10× Genomics) with the GRCh 38 human genome as a reference. The gene-cell unique molecular identifier matrix was analyzed using R software version 4.3.3 and Seurat (version 5.1.0). We filtered out low-quality cells expressing mitochondrial genes in > 15% of their total gene expression, < 200 genes, or > 10,000 genes. After quality control, the dataset comprised 118,822 cells with 32,864 genes. Next, we performed standard normalization for the gene expression of each cell based on the total read count and identified highly variable genes ($n=2000$). The dataset was scaled (ScaleData function), and principal component analysis (PCA) (RunPCA function) was performed for dimensional reduction. Finally, the cells underwent unsupervised clustering (FindClusters function, resolution = 0.1) and were visualized by Uniform Manifold Approximation and Projection (UMAP), using the top 30 principal components (PCs) (RunUMAP function). To identify

marker genes, upregulated genes in each cluster relative to the other clusters were selected based on the Wilcoxon rank-sum test in Seurat's implementation, with >0.25 log-fold change compared with the other clusters, and a Bonferroni-adjusted $p < 0.05$. Some clusters characterized by similar marker genes were manually combined into one cell type. To identify differentially expressed genes (DEGs), we used the MAST algorithm in Seurat's implementation based on a Bonferroni-adjusted $p < 0.05$ and a $\log_2$-fold change >0.25. The expression levels of *CST1* were compared between T2 and non-T2 CRS epithelial cell clusters. Additionally, the dataset GSE72713 [36] from the NCBI Gene Expression Omnibus database was analyzed. To identify DEGs, normalized read counts were analyzed using DESeq2 [37]. DEGs were defined by a $p < 0.05$, and $\log_2$-fold change >1. Data were visualized as volcano plots using R and the ggplot2 package [38].

3 | Statistical Analysis

Statistical analyses were performed using R (version 4.3.3), GraphPad Prism (version 10.2.3; GraphPad Software, San Diego, CA, USA), and SPSS (version 23.0; IBM Corporation, Armonk, NY, USA). The Spearman's rank correlation test was performed to analyze the correlation between ELF cystatin SN levels and clinical parameters. Categorical variables were analyzed using Fisher's exact test or the chi-square test. The Mann–Whitney U test was used to compare data between two unpaired groups. For multiple comparisons, the Kruskal–Wallis test with Dunn's multiple-comparison test was used. Areas under the curve (AUCs) were compared using MedCalc for Windows (version 15.2; MedCalc Software, Ostend, Belgium). *p*-values less than 0.05 were considered statistically significant.

4 | Results

4.1 | Demographic and Clinical Characteristics of the Study Cohort

Cohort 1 included 53 patients with CRS and 14 control individuals. Patients were classified into T2 CRS ($n = 28$) and non-T2 CRS ($n = 25$) groups based on their tissue eosinophil counts (Table 1; Figure S1). No significant differences in demographic factors and frequencies of nasal polyps (NPs), asthma comorbidity, and atopy were found between the two groups. Patients with T2 CRS had significantly higher Lund-Mackay CT scores and JESREC scores than those with non-T2 CRS. Furthermore, patients with T2 CRS tended to have higher olfactory VAS scores, indicating poorer olfactory function.

4.2 | Analysis of Public RNA-Seq Data Identifies Epithelial Cell-Derived *CST1* as a T2 CRS-Specific Biomarker Candidate

To identify biomarker candidates specifically enriched in T2 CRS, we analyzed publicly available scRNA-seq data from both T2 and non-T2 CRSwNP [35]. Following the initial quality control, unsupervised clustering analysis revealed 11 distinct cell types (Figure 1A). Each cell type was identified based on the expression of cell-type annotation marker genes (Figure 1B, Figure S2). To identify markers detectable in ELF, the DEGs between epithelial cells from each endotype were analyzed. The top DEGs between T2 and non-T2 CRS, as well as those between T2 CRS and the combined non-T2 CRS and control groups, included *CST1*, *CCL26*, *POSTN*, *NTRK2*, and *ALOX15* (Figure 1C, Table S1). *CST1* showed the highest fold-change in expression in the T2 CRS group compared to the other groups (Figure 1C,D, Table S1). *CST1* was almost exclusively expressed in the epithelial cells of T2 CRS (Figure 1E). Analyses of other publicly available bulk RNA-seq data (GSE72713) from the nasal tissues of both T2 and non-T2 CRSwNP also showed that *CST1* expression was exclusively observed in T2 CRS but not in non-T2 CRS and controls (Figure 1F,G). Collectively, these results indicate that *CST1* is a candidate for an epithelial-derived biomarker closely associated with T2 CRS.

4.3 | Cystatin SN Protein is Highly Expressed in ELF From Patients With T2 CRS

To validate our bioinformatic analysis of the transcriptomic data, we analyzed the expression of cystatin SN encoded by *CST1*. The expression level of cystatin SN protein in the nasal tissue homogenates was significantly higher in the T2 CRS group than in the non-T2 CRS and control groups (Figure 2A). Immunofluorescence staining showed that cystatin SN was expressed in $EpCAM^+$ epithelial cells from patients with T2 CRS but not in those with non-T2 CRS (Figure 2B). These findings indicate that cystatin SN was localized specifically in the epithelium of T2 CRS.

Given that ELF contains soluble mediators from epithelial cells at the mucosal surface [31], we next analyzed the expression of cystatin SN in ELF from patients with CRS and control individuals. Similar to the results for nasal tissue homogenates, ELF cystatin SN levels were significantly higher in patients with T2 CRS than in patients with non-T2 CRS and control individuals (Figure 2C). Furthermore, the expression level of ELF cystatin SN significantly correlated with that of tissue cystatin SN (Figure 2D), indicating that ELF can reflect the nasal tissue microenvironment in patients with CRS in terms of cystatin SN expression.

To evaluate whether cystatin SN was secreted toward the apical side of the nasal epithelium, we conducted experiments using HNECs cultured on transwell inserts (Figure 2E). Since IL-4 stimulation induces cystatin SN production from nasal epithelial cells, we stimulated HNECs with IL-4 and then evaluated cystatin SN secretion. Following IL-4 stimulation, we found that cystatin SN levels were increased and detectable in the apical supernatants of the transwell system (Figure 2F), verifying that cystatin SN can be secreted toward the apical side of the nasal epithelium.

Overall, we confirmed that cystatin SN expression is detectable in ELF and that its levels are significantly higher in ELFs of T2 CRS than those of non-T2 CRS.

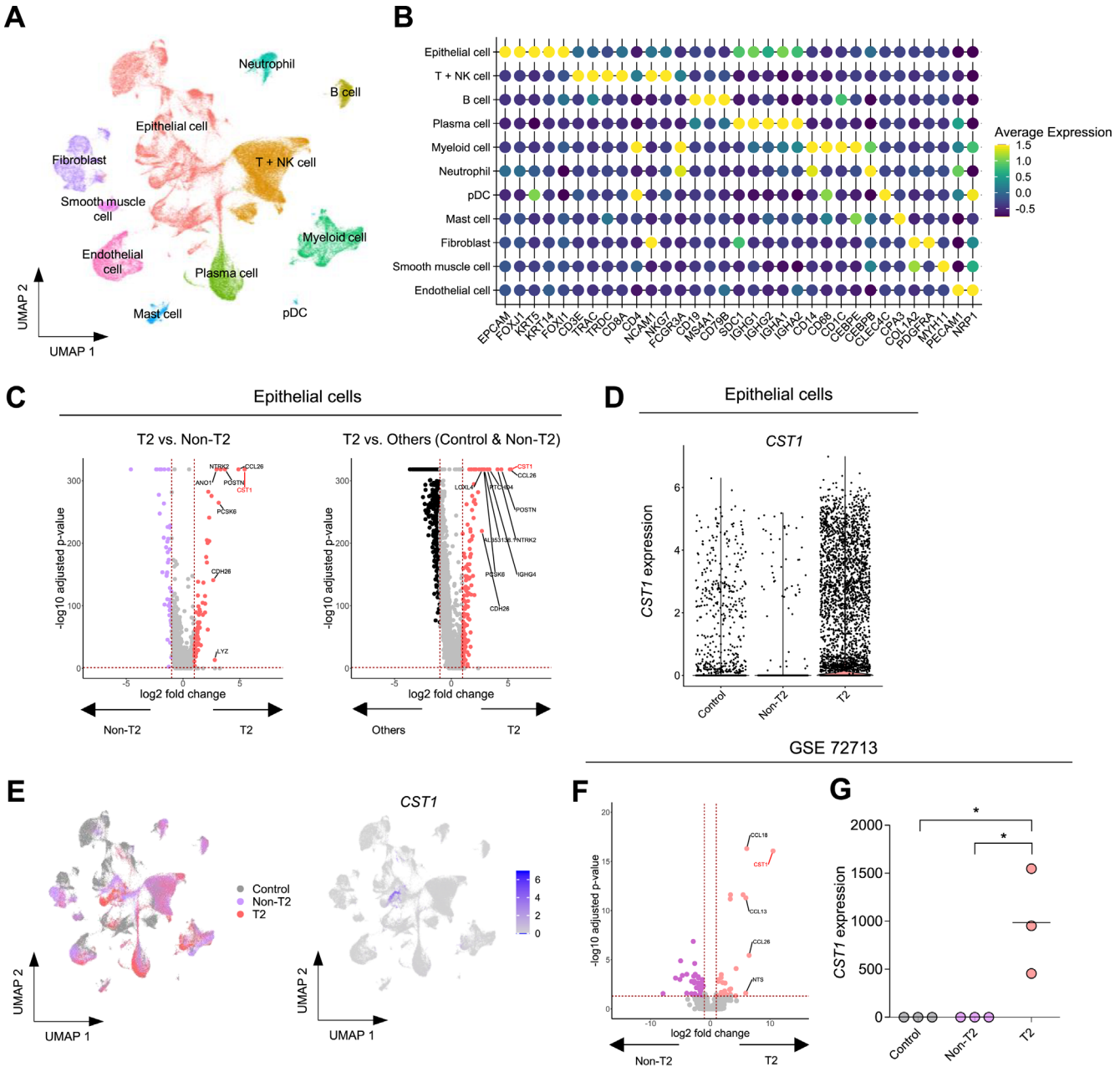

FIGURE 1 | Bioinformatic analysis of public transcriptomic datasets for CRS. (A–E) Publicly available scRNA-seq data (Genome Sequence Archive HRA000772) were analyzed. (A) UMAP projections of 118,822 cells, colored according to each cell type. (B) A dot plot showing expression of cell-type annotation marker genes in each cell cluster. (C) Volcano plots showing differentially expressed genes (DEGs; $\log_2$-fold change > 1 and adjusted $p < 0.05$) in epithelial cells between T2 and non-T2 CRS, and between T2 CRS and the combined non-T2 CRS and control groups. (D) The expression level of *CST1* in epithelial cells from each patient group. (E) UMAPs illustrating *CST1* expression in the epithelial cells of T2 CRS. (F, G) Analysis of the public bulk RNA-seq dataset (GSE72713). (F) A volcano plot showing DEGs between T2 and non-T2 CRS. (G) The expression level of *CST1* in each patient group. Statistical analysis was performed using the one-way ANOVA with Tukey's multiple-comparisons test (G). *$p < 0.05$.

4.4 | High ELF Cystatin SN Expression is Associated With Severe Disease and Poor Olfactory Function in Patients With CRS

We further investigated the relationship between ELF cystatin SN levels and the clinical characteristics of the patients. The cystatin SN concentration in the ELF was positively correlated with the Lund-Mackay CT score ($p = 0.0004$, $R = 0.4714$) and SNOT-22 score ($p = 0.0105$, $R = 0.3486$) (Figure 3A,B). In contrast, ELF cystatin SN levels were inversely correlated with the TDI score ($p = 0.0102$, $R = -0.3535$; Figure 3C) and positively correlated with olfactory VAS score ($p = 0.0029$, $R = 0.4052$) (Figure 3D). ELF cystatin SN levels significantly correlated with the JESREC score ($p = 0.0003$, $R = 0.4796$; Figure 3E). Additionally, when we divided the patients into two groups based on the JESREC score, the ELF cystatin SN levels were significantly higher in the eosinophilic CRS (ECRS) group than in the non-ECRS group (Figure 3F). Other parameters, including sex, age, BMI, and asthma comorbidity, did not correlate

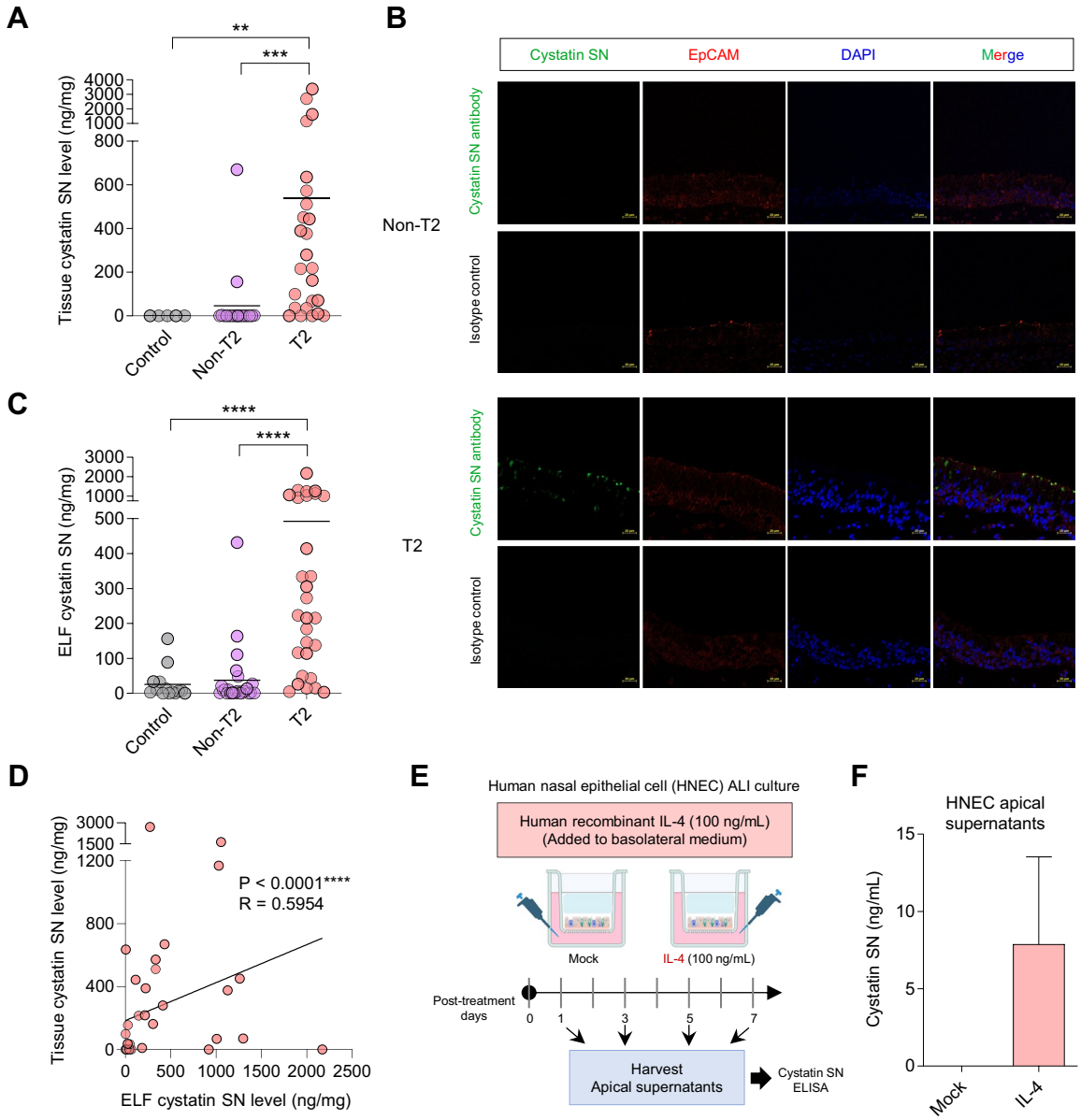

FIGURE 2 | Cystatin SN expression in nasal tissue and ELF. (A) The expression level of cystatin SN in nasal tissue homogenates from controls ($n = 5$), non-T2 CRS ($n = 18$), and T2 CRS ($n = 24$). (B) Representative immunofluorescence images showing expression of cystatin SN and EpCAM in the nasal tissue of T2 CRS and non-T2 CRS. (C) The expression level of cystatin SN in ELF from controls ($n = 14$), non-T2 CRS ($n = 25$), and T2 CRS ($n = 28$). (D) Correlation between the expression level of cystatin SN in ELF and its level in paired nasal tissue homogenates among patients with CRS ($n = 42$). (E and F) HNECs were cultured with or without recombinant human IL-4 (100 ng/mL) for 7 days. Then the apical supernatant was collected, and cystatin SN expression was measured. (E) Experimental scheme. (F) Summary data showing cystatin SN expression levels in apical supernatants from HNEC cultures ($n = 3$). Statistical analysis was performed using the Kruskal–Wallis test with Dunn's multiple comparisons test (A, C) or the Spearman's rank correlation test (D). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

with ELF cystatin SN concentrations. These findings indicate that higher levels of cystatin SN in ELF are associated with increased disease severity, as measured by CT and symptom scores, and decreased olfactory function.

4.5 | ELF Cystatin SN Expression Correlates With T2 Inflammation in the Nasal Tissue

As cystatin SN expression was exclusively observed in epithelial cells from T2 CRS, and its expression levels correlated with the JESREC score and poor olfactory function, we next investigated whether the expression of cystatin SN in ELF reflected T2 inflammation in the nasal tissue.

ELF cystatin SN levels positively correlated with tissue eosinophil count ($p = 0.0013$, $R = 0.4956$), blood eosinophil percentage ($p < 0.0001$, $R = 0.5213$), and blood eosinophil count ($p = 0.0005$, $R = 0.4615$) (Figure 4A), implying that ELF cystatin SN levels correlate with eosinophilic inflammation in patients with CRS. We next evaluated the tissue microenvironment by measuring the expression of T1 cytokine IFN-γ, T2 cytokine IL-5, T3 cytokine IL-17A, and chemokines related to eosinophil chemotaxis, including eotaxin-2 (CCL24) and eotaxin-3

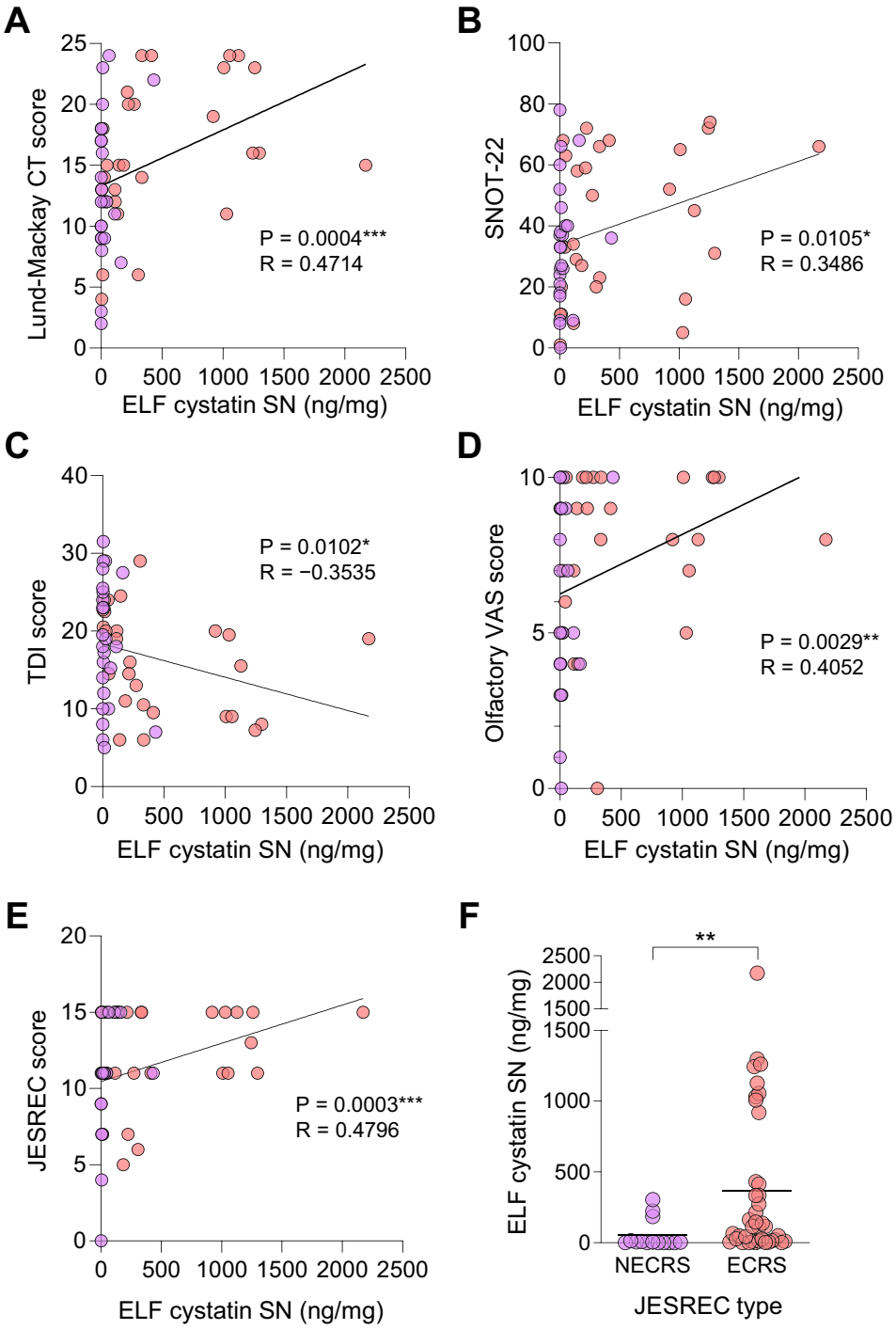

FIGURE 3 | Correlation of ELF cystatin SN expression with clinical characteristics. Correlation of ELF cystatin SN levels with (A) the Lund-Mackay CT score, (B) SNOT-22 score, (C) TDI score, (D) olfactory VAS score, and (E) JESREC score. (F) Patients were divided into two groups based on the JESREC score: Eosinophilic CRS (ECRS; $n = 39$) and non-ECRS ($n = 14$). The ELF cystatin SN levels were compared between the two groups. Statistical analysis was performed using Spearman's rank correlation test (A–E) or the Mann–Whitney U test (F). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(CCL26), in the nasal tissue homogenates. Expression levels of IL-5, eotaxin-2, and eotaxin-3 were significantly higher in T2 CRS than in non-T2 CRS, whereas those of IFN-γ and IL-17A did not differ between the groups (Figure 4B). We further examined the correlation between ELF cystatin SN levels and the expression levels of these markers in the nasal tissue. Cystatin SN levels in ELF positively correlated with the tissue levels of IL-5 ($p = 0.0289$, $R = 0.3992$), eotaxin-2 ($p = 0.0033$, $R = 0.5186$), and eotaxin-3 ($p = 0.0005$, $R = 0.6000$), whereas those negatively correlated with tissue IFN-γ levels ($p = 0.0099$, $R = -0.4634$) (Figure 4C). No correlation was observed between ELF cystatin SN level and tissue IL-17A level ($p = 0.3461$, $R = -0.1782$) (Figure 4C). Similar findings were observed when patients with CRS were subdivided according to IL-5 expression in tissue homogenates, revealing significantly higher ELF cystatin SN levels in patients with T2 CRS

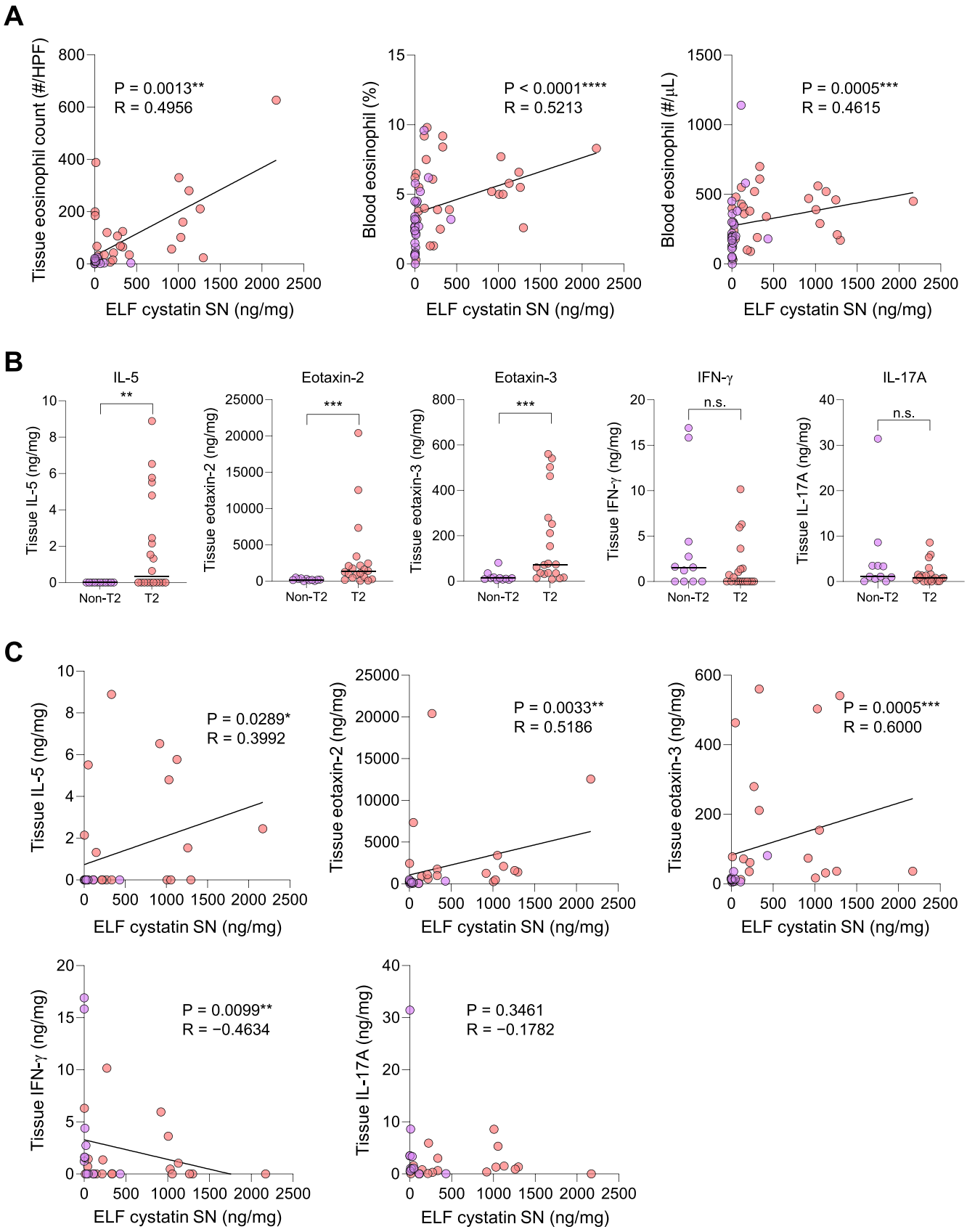

FIGURE 4 | Correlation between ELF cystatin SN expression and the expression of inflammatory mediators in nasal tissue. (A) Correlation of ELF cystatin SN levels with tissue eosinophil count, blood eosinophil percentage, and blood eosinophil count ($n = 53$). (B) Expression levels of IL-5, eotaxin-2, eotaxin-3, IFN-γ, and IL-17A in nasal tissue homogenates of patients with T2 CRS ($n = 19$) and those with non-T2 CRS ($n = 11$). (C) Correlation between ELF cystatin SN levels and tissue levels of IL-5, eotaxin-2, eotaxin-3, IFN-γ, and IL-17A ($n = 30$). Statistical analysis was performed using Spearman's rank correlation test (A, C) or the Mann–Whitney *U* test (B). n.s. not significant, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

(IL-5$^+$) compared to non-T2 CRS (IL-5$^-$) (Figure S3). Overall, these results demonstrated that ELF cystatin SN levels reflect T2 inflammation in the nasal tissue.

4.6 | ELF Cystatin SN Levels Predict T2 CRS With High Accuracy

Next, we evaluated the efficacy of ELF cystatin SN as a biomarker for predicting T2 CRS by generating a receiver operating characteristic (ROC) curve. The analysis revealed an AUC of 0.894, indicating that ELF cystatin SN was an efficient biomarker for discriminating T2 CRS from non-T2 CRS (Figure 5A). With a cut-off value of 112.5 ng/mg, ELF cystatin SN yielded a 75.0% sensitivity and 92.0% specificity (Table 3). Moreover, among the 23 patients with ELF cystatin SN ≥ 112.5 ng/mg (predicted T2 CRS), 21 (91.3%) were confirmed to have T2 CRS (Figure 5B). ELF cystatin SN also showed high accuracy for distinguishing T2 CRS from controls and non-T2 CRS (AUC = 0.897, $p < 0.0001$; Figure 5C), exhibiting a 75.0% sensitivity and 92.3% specificity with a cut-off value of 112.5 ng/mg (Table 3).

As the EPOS 2020 and EPOS/EUFOREA updates have suggested blood eosinophil count and serum total IgE level as parameters for determining T2 CRS [3, 24], we additionally compared the predictive accuracy of ELF cystatin SN and these markers for distinguishing T2 CRS from non-T2 CRS. The AUC for ELF cystatin SN (AUC = 0.894) was higher than that for blood eosinophil count (AUC = 0.703), blood eosinophil percentage (AUC = 0.749), serum total IgE level (AUC = 0.600), and JESREC score (AUC = 0.687; Figure 5D). Similarly, the AUC for ELF cystatin SN (AUC = 0.897) showed greater accuracy in distinguishing T2 CRS from controls and non-T2 CRS than blood eosinophil count (AUC = 0.732), blood eosinophil percentage (AUC = 0.766), or serum total IgE level (AUC = 0.628; Figure 5E).

We further investigated the accuracy of ELF cystatin SN for predicting T2 CRS in the validation cohort (Cohort 2, $n = 66$). ELF cystatin SN levels were significantly higher in patients with T2 CRS compared to non-T2 CRS and control individuals (Figure 6A). Moreover, the previously established ELF cystatin SN cut-off value (112.5 ng/mg) continued to yield strong performance in the validation cohort, with a 92.9% positive predictive value (Figure 6B). ROC curve analysis further validated the predictive accuracy of ELF cystatin SN, showing AUC values of 0.936 ($p < 0.0001$) for distinguishing T2 CRS from non-T2 CRS (Figure 6C) and 0.939 ($p < 0.0001$) for distinguishing T2 CRS from both non-T2 CRS and controls (Figure 6D). From these results, we can conclude that ELF cystatin SN is a noninvasive biomarker with superior accuracy in predicting T2 CRS.

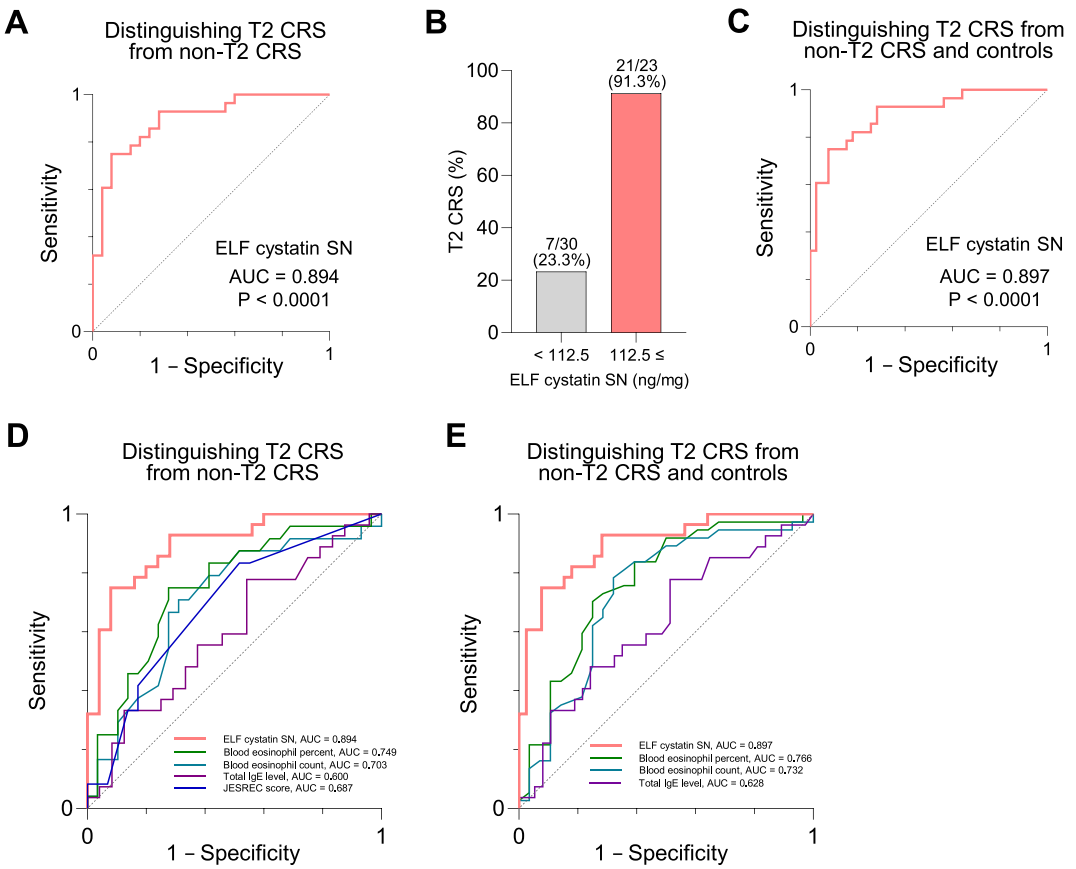

FIGURE 5 | The efficacy of ELF cystatin SN in predicting T2 CRS. (A) A ROC curve for ELF cystatin SN distinguishing T2 CRS from non-T2 CRS. (B) Proportion of patients with T2 CRS among those with ELF cystatin SN of ≥ 112.5 ng/mg ($n = 23$) and < 112.5 ng/mg ($n = 30$). (C) A ROC curve for ELF cystatin SN distinguishing T2 CRS from controls and non-T2 CRS. (D) A combined ROC curve for ELF cystatin SN, blood eosinophil count, blood eosinophil percentage, serum total IgE, and JESREC score distinguishing T2 CRS from non-T2 CRS. (E) A combined ROC curve for ELF cystatin SN, blood eosinophil count, blood eosinophil percentage, and serum total IgE distinguishing T2 CRS from controls and non-T2 CRS.

4.7 | Analysis of the Predictive Value of ELF Cystatin SN Using Alternative Criteria for Defining T2 CRS

We performed additional analyses, in which we used alternative criteria to define T2 CRS in Cohort 1. When defining T2 CRS by a tissue eosinophil count of > 55 cells/HPF, we similarly detected significantly higher ELF cystatin SN levels in patients with T2 CRS compared to non-T2 CRS and control individuals (Figure S4A). ROC curve analysis yielded good predictive accuracy (AUC = 0.770, $p = 0.0047$; Figure S4B), with a higher ELF cystatin SN cut-off value (248.1 ng/mg, sensitivity 68.8%, specificity 87.0%) compared to our original analysis. When defining T2 CRS by a blood eosinophil count of > 150 cells/μL, ELF cystatin SN levels were also significantly higher in T2 CRS compared to non-T2 CRS and control individuals (Figure S4C). Similarly, ROC curve analysis yielded good predictive accuracy (AUC = 0.740, $p = 0.0149$; Figure S4D) with a cut-off value of 14.6 ng/mg (sensitivity 71.4%, specificity 81.8%). Altogether, these results indicate that ELF cystatin SN is a feasible biomarker for predicting T2 CRS, although its cut-off value and predictive accuracy may vary depending on the definition and severity of T2 inflammation.

TABLE 3 | Cut-off value and diagnostic accuracy of each biomarker for identifying T2 CRS in Cohort 1 (discovery cohort).

	Cut-off value	Sensitivity (%)	Specificity (%)
T2 CRS from non-T2 CRS			
ELF cystatin SN (ng/mg)	112.5	75.0	92.0
Blood eosinophil count (cells/μL)	230.0	70.8	69.0
Blood eosinophil percent (%)	3.6	75.0	72.4
Total IgE level (IU/mL)	70.1	77.8	45.8
JESREC score	12.0	83.3	48.3
T2 CRS from non-T2 CRS and controls			
ELF cystatin SN (ng/mg)	112.5	75.0	92.3
Blood eosinophil count (cells/μL)	285.0	78.4	67.9
Blood eosinophil percent (%)	3.7	70.3	75.0
Total IgE level (IU/mL)	70.0	77.8	48.7

Abbreviations: CRS, chronic rhinosinusitis; ELF, epithelial lining fluid; JESREC, Japanese epidemiological survey of refractory eosinophilic chronic rhinosinusitis.

5 | Discussion

In the present study, we identified ELF cystatin SN as a noninvasive and clinically useful biomarker for distinguishing T2 CRS from non-T2 CRS. Our findings demonstrated that the expression level of cystatin SN was significantly elevated in the ELF from patients with T2 CRS and correlated with various clinical and T2 inflammatory markers. ROC curve analysis further corroborated its utility as a T2-specific biomarker.

In recent years, the need for clinically applicable biomarkers has been emphasized [39]. Several methods have been introduced for sampling soluble proteins in the fluid lining the surface of the nasal mucosa [40]. Nasal lavage or irrigation with saline is the most popular method and has been extensively used in previous studies. However, nasal lavage fluid collection has some limitations, including patient discomfort due to the irrigation and collection procedure, the possibility of excessive dilution, variability in sampling quantity, and difficulties in standardization. Nasal aspiration also presents challenges, including the difficulty of accurately assessing the dilution factor, which limits precise biomarker quantification. Additionally, collecting secretions through nose blowing or dripping often results in

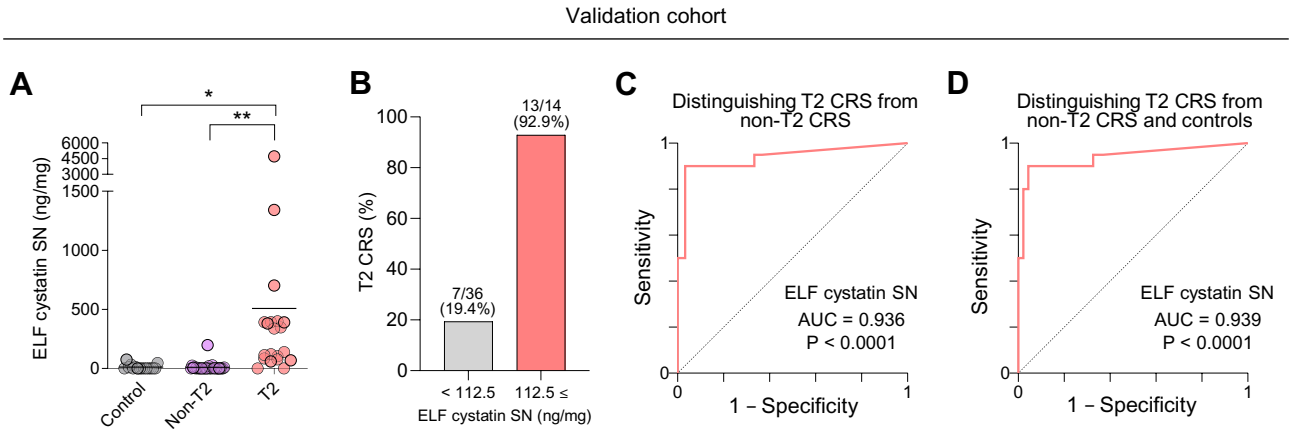

FIGURE 6 | Analysis of the validation cohort. (A) Cystatin SN expression levels in ELF from patients with non-T2 CRS ($n = 30$) and T2 CRS ($n = 20$), and controls ($n = 16$). (B) Proportion of patients with T2 CRS among those with ELF cystatin SN of ≥ 112.5 ng/mg ($n = 14$) and < 112.5 ng/mg ($n = 36$). (C and D) ROC curves for the use of ELF cystatin SN to distinguish T2 CRS from non-T2 CRS (C), or controls and non-T2 CRS (D). $^{*}p < 0.05$, $^{**}p < 0.01$.

inconsistent sampling and variable sample quality. In contrast, the presently used ELF sampling method using Leukosorb paper offers several advantages, including minimal dilution of soluble biomarkers, targeted sampling from specific anatomical sites, minimal patient discomfort, and well-standardized sampling quantities. This sampling technique is feasible for performance in outpatient clinical settings.

The use of ELF biomarkers offers several advantages over current diagnostic methods. The traditional method for diagnosing T2 CRS involves histopathological evaluation to assess eosinophil infiltration following invasive procedures such as biopsy and surgery. Although accurate, these methods are time-consuming and difficult to perform in routine clinical practice. Additionally, although the top DEGs in T2 CRS compared to non-T2 CRS in transcriptomic analyses have been suggested as potential biomarkers [17], analyzing transcript levels in NP tissue also requires invasive procedures, and the combination of several markers is complex, making it challenging to translate into clinical practice. In contrast, measuring the protein levels of a single biomarker in ELFs is a less invasive, rapid, and simple alternative. As cystatin SN is a secretory protein and was predominantly expressed in the epithelial cells of T2 CRS in scRNA-seq analysis, it was selected as an ELF biomarker candidate. Cystatin SN expression was detected in the ELF from patients with T2 CRS. The significant correlation in cystatin SN concentration between ELF and tissue homogenate indicates that ELF is an indirect but reliable method for measuring nasal cystatin SN expression. Our ROC curve analysis showed that ELF cystatin SN had a higher AUC than other peripheral blood-derived, noninvasive biomarkers such as blood eosinophil count and serum total IgE level, indicating its superior accuracy in diagnosing CRS. Although previous studies have shown a close relationship between cystatin SN and T2 CRS, this is the first study to evaluate the feasibility of measuring cystatin SN expression in ELFs to diagnose T2 CRS in comparison with existing biomarkers.

Cystatin SN belongs to the type 2 cystatin protein superfamily and functions as a cysteine protease inhibitor [41]. It is expressed on the nasal epithelial surface and controls cysteine proteases that are widely expressed in allergens, viruses, and bacteria, thereby protecting against inflammatory tissue remodeling. Previous studies performing transcriptomic and proteomic analyses have reported dysregulated expression of cystatin SN in various airway diseases, including CRS and asthma [41]. High levels of cystatin SN were associated with severe disease symptoms and a poorly controlled status in patients with CRS [42, 43]. Consistent with previous studies, our results demonstrated a significant correlation between ELF cystatin SN levels and disease severity as measured by Lund–Mackay CT and SNOT-22 scores.

Several studies have shown that the cystatin SN is closely associated with eosinophilic inflammation. The expression of cystatin SN in nasal tissues was significantly higher in eosinophilic CRSwNP than in the controls and noneosinophilic CRSwNP [44, 45]. The robust link was further evidenced by the positive correlation of cystatin SN expression with tissue eosinophil percentage and fractional exhaled nitric oxide [42, 45]. In the current study, the scRNA-seq analysis of epithelial cells revealed that *CST1* was the top DEG between T2 CRS and non-T2 CRS. Both immunofluorescence staining and ELISA assays of nasal tissues confirmed the heightened expression of cystatin SN in T2 CRS. Because olfactory dysfunction is more prevalent in T2 CRS than in non-T2 CRS [14], the inverse correlation between ELF cystatin SN levels and olfactory function measures also supports a close relationship between cystatin SN and T2 CRS.

Mechanistically, cystatin SN appears to play a crucial role in amplifying T2 inflammation in CRS. In vitro treatment with cystatin SN upregulated the expression of CCL11/eotaxin-1 and periostin in nasal fibroblasts [44]. Additionally, cystatin SN induced the recruitment and activation of eosinophils through IL-5 [45]. A recent study further showed that exposure to cystatin SN induced the upregulation of T2 cytokines, such as IL-4, IL-5, and IL-13, as well as the infiltration of T helper 2 cells in healthy murine nasal mucosa [42]. Similarly, we found that cystatin SN concentrations in ELF significantly correlated with expression levels of T2 inflammatory mediators (IL-5, eotaxin-2, and eotaxin-3), while negatively correlating with IFN-γ levels. These findings collectively suggest that cystatin SN contributes to the skewing of the inflammatory response toward the T2 endotype. In contrast, a protective role of cystatin SN in allergic airway diseases has also been reported. Cystatin SN suppressed allergic rhinitis symptoms by inhibiting allergen protease activity and protecting the nasal tight junction barrier in an allergen-specific manner [46]. Similar results were observed in a house dust mite-induced asthma model, in which cystatin SN protected the bronchial epithelial barrier by inhibiting allergenic protease activity [47]. However, current understanding of the mechanisms underlying the amplifying effects of cystatin SN on T2 inflammation is limited. Further delineation of the functional roles and regulatory mechanisms of cystatin SN in diverse inflammatory conditions is required to develop novel therapeutic strategies targeting cystatin SN in T2 CRS.

ELF cystatin SN showed promising predictive accuracy for identifying T2 CRS; however, we observed slight discrepancies when using alternative criteria to define T2 CRS, suggesting that its cut-off values and corresponding predictive performance are influenced by the definition and severity of T2 inflammation. External validation in larger multi-institutional cohorts is required to firmly establish the diagnostic accuracy and clinical applicability of ELF cystatin SN across diverse patient populations.

In summary, our current investigation highlights ELF cystatin SN as a promising and useful predictive biomarker for T2 CRS. High expression in patients with T2 CRS, robust correlation with clinical and T2 inflammatory markers, and superior diagnostic accuracy compared to existing noninvasive markers make ELF cystatin SN a valuable tool for CRS endotyping. The use of ELF cystatin SN levels as a noninvasive biomarker may facilitate more personalized treatment, consequently improving treatment outcomes and reducing the disease burden of CRS.

Author Contributions

S.M. (Seojin Moon) designed the study, analyzed the data, and wrote the manuscript; S.M. (Sungmin Moon) performed the experiments, analyzed the data, and wrote the manuscript; S.L., G.K., and M.-S.K. performed the experiments and analyzed the data; H.-J.C. and C.-H.K. provided clinical samples and analyzed the data; M.-S.R. designed the

study, provided clinical samples, analyzed the data, wrote the manuscript, and reviewed the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.