

Letter to the Editor

Heterogeneous inflammatory patterns in chronic rhinosinusitis without nasal polyps in Chicago, Illinois

To the Editor:

Although it is accepted that chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP) is characterized by type 2 inflammation with pronounced eosinophilia and the presence of high levels of IL-5 and IL-13 in Western countries, the mechanism of inflammation in patients with chronic rhinosinusitis without nasal polyps (CRSSNP) is poorly understood.^{1,2} Initial studies by Van Zele et al³ in Belgium suggested that CRSSNP is characterized by type 1 inflammation on the basis of elevation in IFN- γ levels. Although several articles from the same group have confirmed these findings,⁴ other groups, including our own, have been unable to find elevation in IFN- γ levels in CRSSNP.^{5,6} We therefore examined potential differences in experimental design between these studies. Studies that found type 1 inflammation in CRSSNP compared inferior turbinate (IT) tissue from controls, ethmoid tissue (ET) from patients with CRSSNP, and nasal polyp (NP) tissue from patients with CRSwNP.^{3,4,7} In contrast, those that did not find type 1 inflammation compared uncinate tissue (UT) from controls, patients with CRSSNP and CRSwNP, and patients with NPs.^{5,6} It was thus unclear whether reported IFN- γ elevations were due to differences in sampled anatomy or countries.

To clarify patterns of inflammatory cytokines in CRSSNP in our study population, we collected IT, UT, and ET from control patients and patients with CRSSNP and CRSwNP (see Fig E1 and Tables E1 and E2 in this article's Online Repository at www.jacionline.org), and determined the presence of IFN- γ by real-time RT-PCR. Detailed methods are given in this article's Online Repository at www.jacionline.org. We found that the IFN- γ level was not significantly elevated in CRSSNP when compared with controls or patients with CRSwNP within the same tissue type (Fig 1, A). We also compared CRSSNP ET to control IT or CRSwNP NP as previously reported.^{3,4} However, the IFN- γ level was not elevated in ET from CRSSNP (Fig 1, B). We also analyzed a marker of eosinophilia, Charcot-Leyden crystal galectin (CLC, also known as eosinophil lysophospholipase), type 2 cytokines, IL-5 and IL-13, and the type 3 cytokine IL-17A. Because we found significant differences in the levels of CLC and IL-17A between control and CRSSNP in ET and not in IT or UT (Fig 1; see Fig E2 in this article's Online Repository at www.jacionline.org), we focused our further analysis on ET. We represent further analysis using dot plots to better illustrate the inflammatory patterns in individual specimens.

On further analysis, CLC, IL-5, and IL-13 levels were expectedly significantly higher in CRSwNP ET compared with control ET (Fig 1, C). However, the levels of CLC, IL-5, and IL-17A were also significantly elevated in CRSSNP ET compared with control ET (Fig 1, C). CLC expression positively correlated with IL-5 ($r = 0.3652$; $P = .0037$) and IL-13 ($r = 0.7262$; $P < .0001$), but not with IL-17A or IFN- γ in the CRSSNP ET ($n = 61$, not shown).

We next established thresholds for defining each inflammatory subtype using the 95th percentile of expression in control ETs.⁸

Using these thresholds, 23%, 36%, and 15% of CRSSNP ET showed type 1, 2, and 3 inflammation, based on the expression of IFN- γ , CLC, and IL-17A, respectively (Fig 1, C). In contrast, CRSwNP ET and NP had higher frequencies of type 2 inflammation (65% and 77%) but lower frequencies of type 1 (10% and 15%) or 3 (8% and 6%) inflammation (Fig 1, C). Interestingly, minor subsets of CRSSNP donors had mixed inflammation: 8% showed type 1 and 2 mixed inflammation, 7% showed type 1 and 3 mixed inflammation, and a single donor showed all 3 types in ETs (see Fig E3 in this article's Online Repository at www.jacionline.org). Also noteworthy was that 43% of CRSSNP donors did not have elevated type 1, 2, or 3 inflammation in ETs (Fig E3). We initially hypothesized that the type of inflammation in CRSSNP might be correlated with clinical comorbidities such as atopy or asthma. However, we found no significant differences in the levels of inflammation markers between atopic and nonatopic patients or between patients with and without asthma in CRSSNP (not shown).

We further confirmed our findings at the protein level. We generated protein tissue extracts from ETs and NPs and measured eosinophil cationic protein (ECP) and cytokine levels by ELISA and Luminex, respectively (see Table E2). Similar to our RT-PCR results, IFN- γ protein level was not elevated but markers of type 2 (ECP, IL-5, and IL-13) and 3 (IL-17A) inflammation were significantly elevated in CRSSNP ET compared with control ET (Fig 2). Using the 95th percentile of protein expression in control ETs to define inflammatory subtypes, we found that 5%, 46%, and 16% of CRSSNP ET showed type 1, 2, and 3 inflammation, based on the expression of IFN- γ , ECP, and IL-17A, respectively (Fig 2). Although the frequency of type 2 and 3 inflammation was similar to that established using mRNA expression, the frequency of type 1 inflammation was lower when using protein measures (Fig 2). One possible explanation is the decreased sensitivity of the IFN- γ protein detection system compared with real-time RT-PCR. We therefore further analyzed the data classifying only by type 2 and 3 inflammation in CRSSNP ET. We found that 37% showed type 2, 7% showed type 3, 8% showed mixed type 2 and 3 inflammation, and 47% of CRSSNP donors showed neither type 2 nor 3 inflammation in ETs (see Fig E4 in this article's Online Repository at www.jacionline.org).

Our study has some limitations. Patient-matched IT, UT, and ET were not available from all patients due to variations in the extent of surgery, the size of surgically resected tissue, and the quality of RNA extracted. Subsequently, the numbers of specimens available from each anatomic location and for analysis by protein or RT-PCR were variable. Our study also recruited patients undergoing surgery in a tertiary care practice in Chicago. Thus, whether our results are applicable to the general population, or only tertiary care populations in the United States, would require further multiinstitutional studies. Nonetheless, we report data from a larger number of samples than do most other published studies and had sufficient control ET to define a 95th percentile threshold for inflammatory subtyping. Our results also suggest that CRSSNP cannot be generalized as a type 1 inflammatory condition because approximately 40% and 15% of patients with CRSSNP demonstrated type 2 and 3 inflammation, respectively. We also further find that more than 40% of patients with CRSSNP did not show the signature for type 1, 2, or

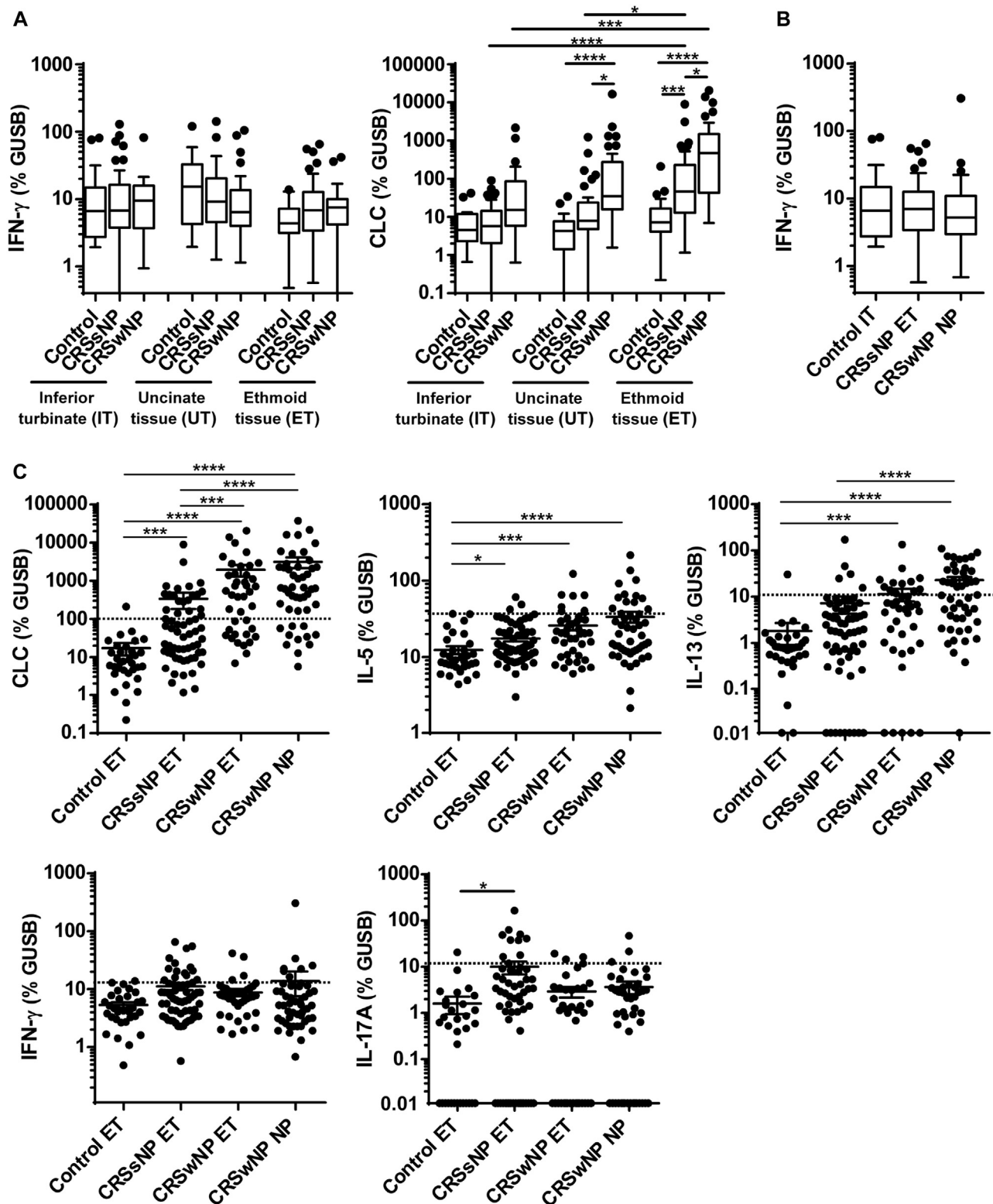


FIG 1. Messenger RNAs for markers of type 2 and 3 inflammation were elevated in patients with CRSsNP. Total RNA was extracted from whole tissue of control IT (n = 19), control UT (n = 21), control ET (n = 33), CRSsNP IT (n = 53), CRSsNP UT (n = 44), CRSsNP ET (n = 61), CRSwNP IT (n = 28), CRSwNP UT (n = 29), CRSwNP ET (n = 40), and CRSwNP NP (n = 48). Expression of mRNAs for IFN- γ , CLC, IL-5, IL-13, and IL-17A was analyzed using real-time RT-PCR. Gene expression was normalized to a housekeeping gene, β -glucuronidase (GUSB), and expression levels were shown as % expression of GUSB. Results are shown as medians (25% to 75% interquartile ranges) (A and B) or mean $\pm$ SEM (C). Dotted line indicates the threshold based on the 95th percentile expression in control ET (CLC, 96.4; IL-5, 36.5; IL-13, 11.2; IFN- γ , 13.2; IL-17A, 12.1) (Fig 1, C). To display undetectable data, we plotted 0 as 0.01 in log-scaled figures (Fig 1, C). * P < .05, *** P < .001, **** P < .0001, by 1-way ANOVA.

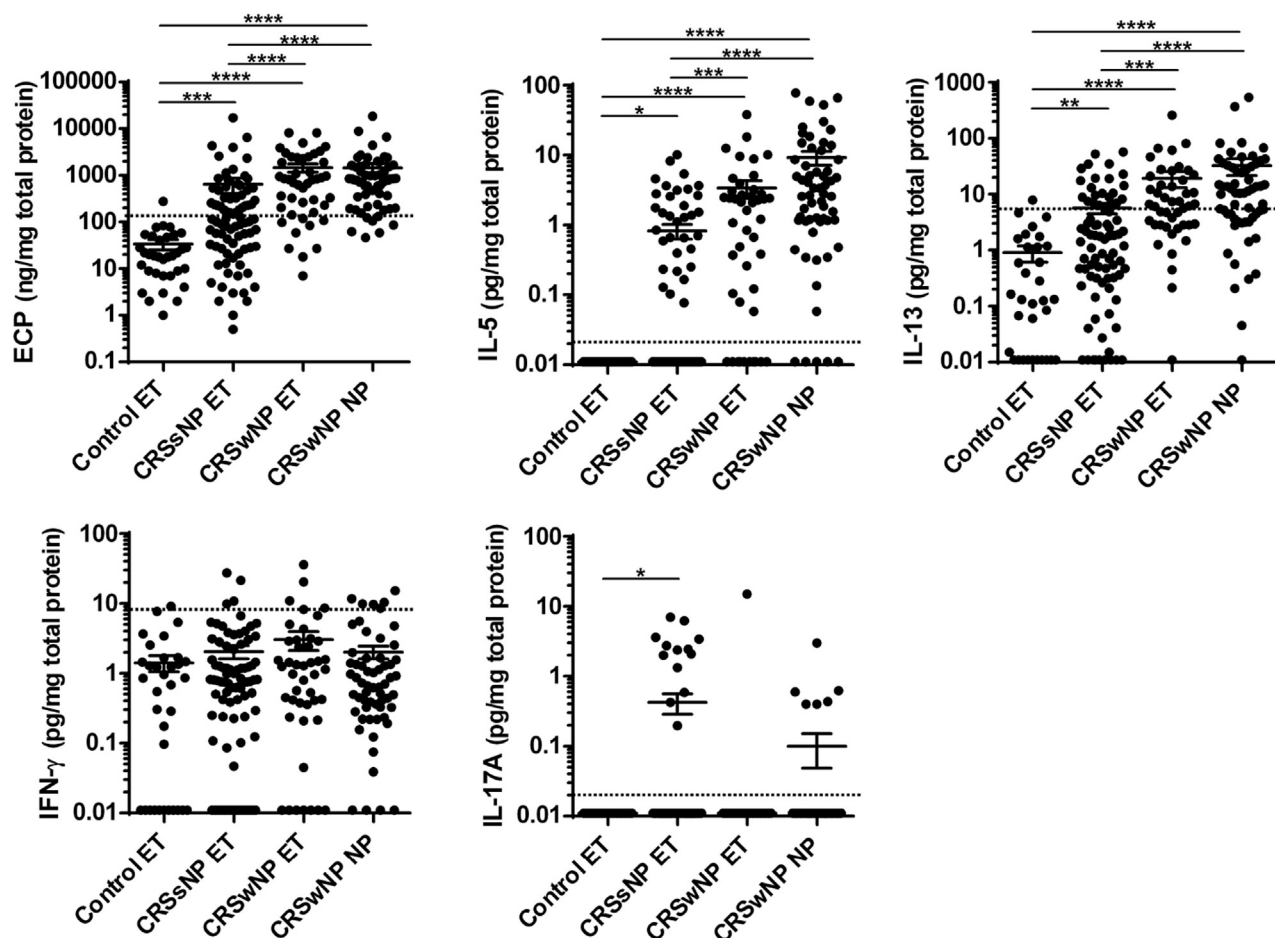


FIG 2. Type 2 and 3 cytokines were elevated in CRSsNP tissue protein extracts. Protein extracts were generated from ET of control ($n = 34$), CRSsNP ($n = 83$), CRSwNP ($n = 45$), and NPs ($n = 60$). Expression of ECP, IL-5, IL-13, IFN- γ , and IL-17A proteins in tissue homogenates was measured using ELISA and Luminex. The protein concentrations were normalized to the concentration of total protein. Dotted line indicates the threshold based on the 95th percentile expression in control ET (ECP, 131.5 ng/mg; IL-5, 0.02 pg/mg [detectable]; IL-13, 5.5 pg/mg; IFN- γ , 8.1 pg/mg; IL-17A, 0.02 pg/mg [detectable]). To display undetectable data, we plotted 0 as 0.01 in log-scaled figures. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$, by 1-way ANOVA.

3 inflammation in ET, IT, or UT (Fig 1; Figs E3 and E4 and not shown). Further studies will be required to identify the nature of the inflammation in these patients.

Using flow cytometry, Derycke et al⁹ have reported heterogeneous T-helper populations in CRSsNP, with T_H1 being the most common population in control, CRSsNP, and CRSwNP populations. T_H2 cells were extremely rare in the CRSsNP tissue studied. In contrast, although we also report heterogeneous inflammatory patterns in CRSsNP, type 2 inflammation was most common. Although Derycke et al⁹ used stimulated cells to identify the presence of T-helper subsets in tissue,⁹ our study evaluated relative ongoing expression of inflammatory cytokines and eosinophil granule proteins. Because these studies use different biomarkers for each inflammatory subtype and have disparate methods for defining an inflammatory subtype, they are not directly comparable. The methodologic differences may indeed lead to the different results.

Very recently, 2 groups published on patterns of inflammation in CRS. In support of our findings, König et al¹⁰ found that the IFN- γ level was not elevated in the nasal secretion of German

patients with CRSsNP. Tomassen et al¹¹ proposed 10 endotypes of CRS on the basis of inflammatory patterns found in a multicenter study in Europe. This study also discovered heterogeneous inflammation in CRSsNP and the overall frequency of IFN- γ , IL-5, and IL-17A high populations in CRSsNP was 20%, 30%, and 11%, respectively, which is similar to that in our current study. Together, these results may indicate that our findings may be applicable to patients with CRSsNP in both the United States and Europe.

In conclusion, we report here that CRSsNP is a heterogeneous disease and the overall frequency of type 2 inflammation is higher than type 1 inflammation in our US-based population. In light of emerging therapies targeting type 2 inflammatory mechanisms, our findings indicate that further studies will be needed to better identify patients with type 2 CRSsNP for tailored therapeutic strategies.

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METHODS

Patients and biopsies

Patients with CRS were recruited from the Otolaryngology Clinic and the Northwestern Sinus Center of Northwestern Medicine. IT tissue, UT, ET, and NP tissue were obtained during routine endoscopic sinus surgery performed on patients with CRS. All patients met the criteria for CRS as defined by the American Academy of Otolaryngology-Head and Neck Surgery Chronic Rhinosinusitis Task Force.^{E1,E2} Patients with an established immunodeficiency, pregnancy, coagulation disorder, or diagnosis of aspirin hypersensitivity, classic allergic fungal sinusitis, Churg-Strauss syndrome, or cystic fibrosis were excluded from the study. Disease-free sinus tissues from normal control patients without a history of CRS were obtained during procedures for conditions other than CRS (septoplasty for nasal obstruction, transnasal endoscopy skull base procedures, repairs of facial fractures, treatment of nasal disorders in obstructive sleep apnea, etc). Patients were skin-tested for pollens, dust mites, pets, molds, and cockroach using Hollister-Stier (Spokane, Wash) extracts. Several patients were taking various medications, including corticosteroids (Tables E1 and E2). Details of patients' characteristics are included in Tables E1 and E2. All patients signed informed consent forms, and the protocol governing procedures for this study was approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine (institutional review board protocol no. STU00016917).

Real-time RT-PCR

A portion of nasal tissues for isolation of RNA was transferred in RNA later (Ambion, Austin, Tex) and stored at -20°C . Total RNA from sinus tissue was extracted using QIAzol (Qiagen, Valencia, Calif) and was cleaned and treated with DNase I using NucleoSpin RNA (Clontech Laboratories, Mountain View, Calif) according to the manufacturer's instructions. The quality of total RNA from sinus tissue was assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, Calif) using a RNA 6000 Nano LabChip (Agilent Technologies). RNA in which RIN was greater than 7.0 was used for cDNA synthesis. Single-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Calif) and random primers. Real-time RT-PCR was performed with a TaqMan method using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, Calif) in 20- μL reactions (10 μL 2 $\times$ TaqMan Fast Advanced Master Mix [Applied Biosystems]), 1 μL 20 $\times$ primer and probe mixture for target gene, 1 μL 20 $\times$ primer and probe mixture for β -glucuronidase (GUSB) plus cDNA equivalent to 10 ng of total RNA. Primer and probe sets for IFN- γ (Hs00989291_m1), CLC (Hs00171342_m1), IL-17A (Hs00936345_m1), IL-5 (sense, 5'-AGCTGCC TACGTGTATGCCA-3'; antisense, 5'-GTGCCAAGGTCTCTTTCACCA-3'; FAM/BHQ1 probe, 5'-CCCCACAGAAATTTCCACAAAGTGCA-3'), IL-13 (sense, 5'-AAGGTCTCAGCTGGGCAGTTTA-3'; antisense, 5'-AAACTGG GCCACCTCGATT-3'; FAM/BHQ1 probe, 5'-CCAGCTTGCATGTCCGA GACACCA-3'), and GUSB (Human β -glucuronidase endogenous control,

PN; 4326320E) were purchased from Applied Biosystems or Integrated DNA Technologies (Coralville, Iowa). The mRNA expression levels were normalized to the expression of a housekeeping gene, GUSB. The expression of GUSB was not significantly different between control ETs and NPs (data not shown).

Tissue homogenates and protein assays

Freshly obtained tissue specimens were weighed, and 1 mL of PBS supplemented with 0.05% Tween 20 (Sigma-Aldrich, St Louis, Mo) and 1% protease inhibitor cocktail (PN; P8340, Sigma-Aldrich) was added for every 100 mg of tissue. The tissue was then homogenized with a Bullet Blender Blue (Next Advance, Averill Park, NY) at setting 7 for 8 minutes at 4°C . After homogenization, the suspension was centrifuged at 4000 rpm for 20 minutes at 4°C and the supernatants were stored at -80°C . Before analysis, tissue homogenates were centrifuged at 16,000g for 15 minutes at 4°C and we used those supernatants for each assay.

The concentration of ECP in cell-free supernatants was determined by a commercial ELISA kit (MBL, Woburn, Mass). The minimal detection limit for this kit is 0.125 ng/mL. The concentrations of IFN- γ , IL-5, IL-13, and IL-17A in cell-free supernatants were measured using a MILLIPLEX MAP Human High Sensitivity T-Cell Panel from EMD Millipore (Billerica, Mass). The minimal detection limits for IFN- γ , IL-5, IL-13, and IL-17A are 0.61, 0.49, 0.24, and 0.73 pg/mL, respectively. The concentration of ECP and cytokines in tissue homogenates was normalized to the concentration of total protein as detected by BCA protein assay kit (ThermoScientific, Rockford, Ill).

Statistics

All data are reported as the median (25%-75% interquartiles) or as the mean $\pm$ SEM. Differences between groups were analyzed using the 1-way ANOVA Kruskal-Wallis Dunn's multiple comparison test. Correlations were assessed using the Spearman rank correlation. All statistical analyses were performed using GraphPad prism 6.07 software (La Jolla, Calif). A *P* value of less than .05 was considered significant. In the log-scaled figures, we plotted 0 as 0.01 although we did statistical analysis using all the raw data including 0 (undetectable).

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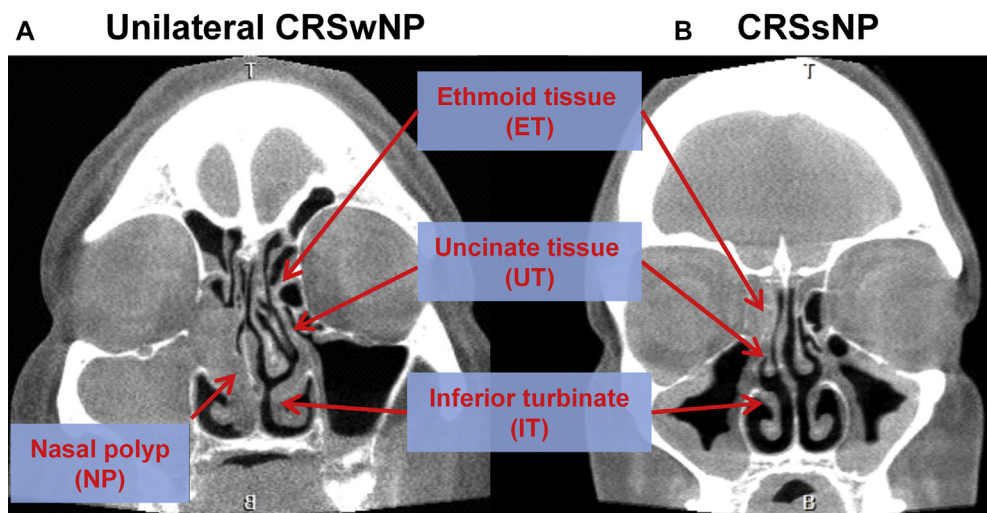


FIG E1. Diagram of nasal and paranasal sinuses. Illustrative computed tomography (CT) images demonstrating the anatomic location of studied tissue from **(A)** a patient with typical changes of CRSwNP on one side but more normal anatomy on the contralateral side and **(B)** a patient with radiographic changes typical for CRSsNP.

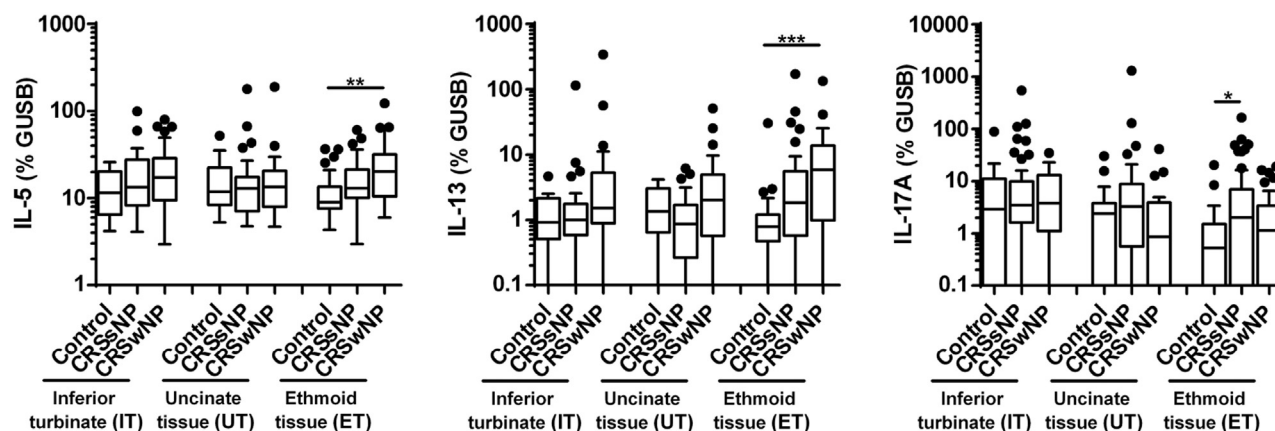


FIG E2. Expression of IL-5, IL-13, and IL-17A in IT, UT, and ET. Total RNA was extracted from whole tissue of control IT (n = 19), control UT (n = 21), control ET (n = 33), CRSsNP IT (n = 53), CRSsNP UT (n = 44), CRSsNP ET (n = 61), CRSwNP IT (n = 28), CRSwNP UT (n = 29), and CRSwNP ET (n = 40). Expression of mRNAs for IL-5, IL-13, and IL-17A was analyzed using real-time RT-PCR. Gene expression was normalized by a housekeeping gene, GUSB, and expression levels were shown as % expression of GUSB. Results are shown as medians (25% to 75% interquartile ranges). * $P < .05$, ** $P < .01$, and *** $P < .001$, by 1-way ANOVA.

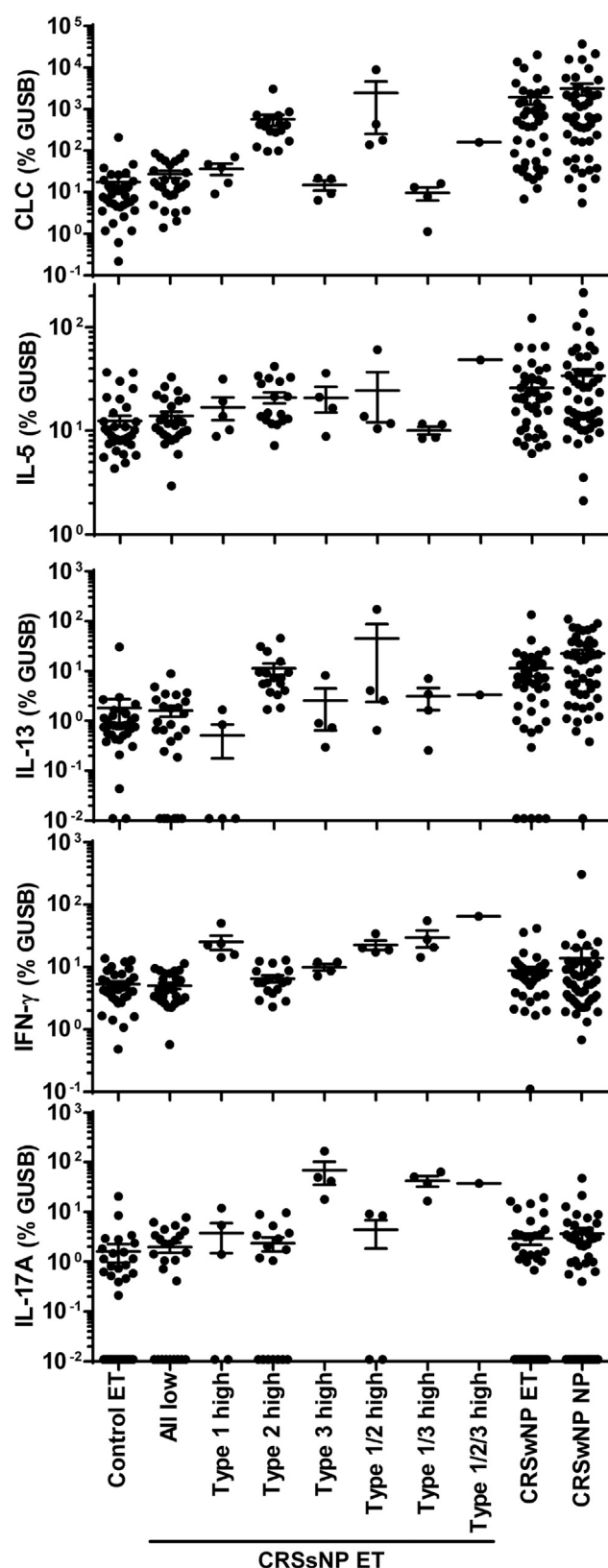


FIG E3. CRSsNP can be divided into several groups. Total RNA was extracted from whole tissue of control ET ($n = 33$), CRSsNP ET ($n = 61$), CRSwNP ET ($n = 40$), and CRSwNP NP ($n = 48$). CRSsNP was further divided into type 1 cytokine high group ($n = 5$), type 2 cytokine high group ($n = 17$), type 3 cytokine high group ($n = 4$), type 1 and 2 cytokines high

group (type 1/2, $n = 4$), type 1 and 3 cytokines high group (type 1/3, $n = 4$), type 1, 2, and 3 cytokines high group (type 1/2/3, $n = 1$), and type 1, 2, and 3 cytokine all low group (all low, $n = 26$). Type 1, 2, and 3 high groups were classified on the basis of 95th percentile expression of IFN- γ (13.2), CLC (96.4), and IL-17A (12.1), respectively, in control ET. Expression of mRNAs for CLC, IL-5, IL-13, and IFN- γ was analyzed using real-time RT-PCR. Gene expression was normalized to a housekeeping gene, GUSB, and expression levels were shown as % expression of GUSB. Results are shown as mean $\pm$ SEM. To display undetectable data, we plotted 0 as 0.01 in log-scaled figures.

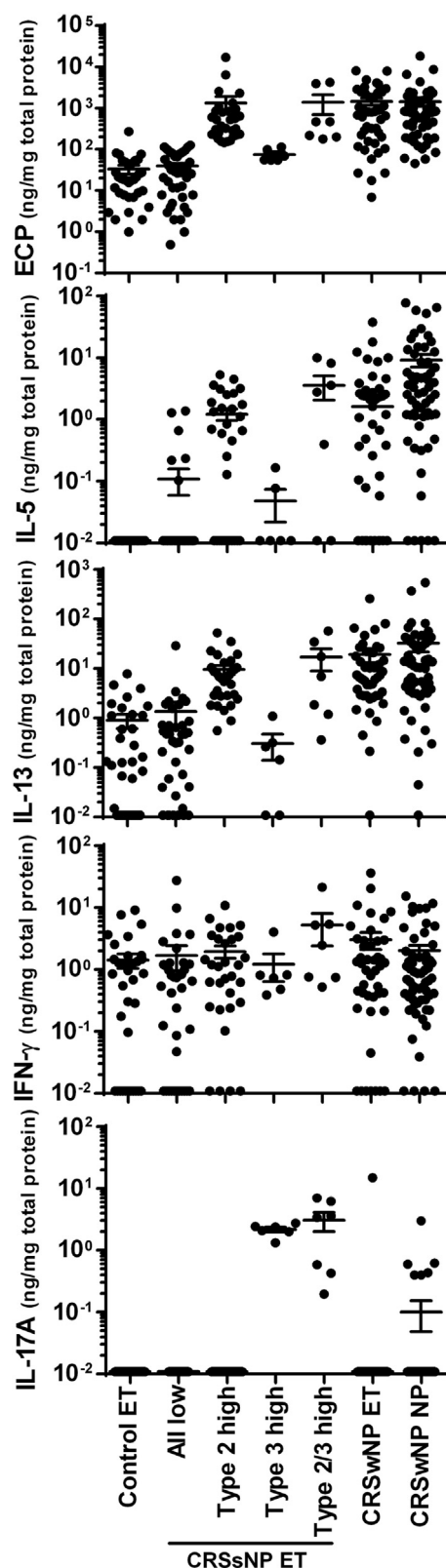


FIG E4. Subclassification of CRSsNP by protein levels of type 2 and 3 cytokines. Protein extracts were generated from whole ET tissues of control ($n = 34$), CRSsNP ($n = 83$), CRSwNP ($n = 45$), and NP tissues ($n = 60$). CRSsNP was further divided into type 2 cytokine high group ($n = 31$), type 3 cytokine high group ($n = 6$), type 2 and 3 cytokines high group (type 2/3, $n = 7$), and type 2 and 3 cytokines all low group (all low, $n = 39$). Expression

of ECP, IL-5, IL-13, IFN- γ , and IL-17A proteins in tissue homogenates was measured using ELISA and Luminex and normalized to the concentration of total protein. Type 2 and 3 high groups were classified on the basis of 95th percentile expression of ECP (131.5 ng/mg) and IL-17A (0.02 pg/mg [detectable]), respectively, in control ET. To display undetectable data, we plotted 0 as 0.01 in log-scaled figures.

TABLE E1. Patients' characteristics in the RNA study

Characteristic	Control IT (n = 19)	Control UT (n = 22)	Control ET (n = 33)	CRSsNP IT (n = 53)	CRSsNP UT (n = 44)	CRSsNP ET (n = 61)	CRSwNP IT (n = 28)	CRSwNP UT (n = 29)	CRSwNP ET (n = 40)	CRSwNP NP (n = 48)
Female	8 (42)	15 (68)	22 (67)	35 (66)	26 (59)	34 (56)	8 (29)	9 (31)	11 (28)	13 (27)
Atopy	2 (11)	3 (14)	7 (21)	21 (40)	18 (41)	29 (48)	13 (46)	14 (48)	22 (55)	29 (60)
Asthma	0 (0)	0 (0)	3 (9)	7 (13)	9 (20)	17 (28)	16 (57)	13 (45)	20 (50)	26 (54)
Nasal steroid	0 (0)	0 (0)	5 (15)	5 (9)	5 (11)	18 (30)	4 (14)	5 (17)	8 (20)	8 (17)
Inhaled steroid	0 (0)	1 (5)	0 (0)	2 (4)	3 (7)	10 (16)	2 (7)	2 (7)	8 (20)	9 (19)
Oral steroid	0 (0)	0 (0)	1 (3)	1 (2)	4 (9)	3 (5)	5 (18)	7 (24)	6 (15)	8 (17)
Age (y), median (range)	36.5* (17-72) [†]	46.5 (16-75)	58.0 (22-75)	37.0 (21-71)	38.5 (20-73)	43.0 (20-70)	45.0 (27-70)	47.0 (23-67)	47.0 (23-72)	45.0 (20-72)

Values are n (%) unless indicated otherwise.

*Median.

[†]Range.

TABLE E2. Patients' characteristics in protein study

Characteristic	Control ET (n = 34)	CRSsNP ET (n = 83)	CRSwNP ET (n = 45)	CRSwNP NP (n = 60)
Female	17 (50)	51 (61)	14 (31)	25 (42)
Atopy	6 (18)	42 (51)	24 (53)	39 (65)
Asthma	4 (12)	32 (39)	19 (42)	28 (47)
Nasal steroid	0 (0)	27 (33)	12 (27)	14 (23)
Inhaled steroid	1 (3)	22 (22)	9 (20)	13 (22)
Oral steroid	1 (3)	12 (14)	9 (20)	6 (10)
Age (y), median (range)	56.5* (22-75) [†]	38 (19-74)	47.0 (24-72)	45.0 (24-76)

Values are n (%) unless indicated otherwise.

*Median.

[†]Range.