

Cytokine Profiles in Japanese Patients with Chronic Rhinosinusitis

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ABSTRACT

Background: Chronic rhinosinusitis (CRS) is classified in CRS without nasal polyp (CRSsNP) and CRS with nasal polyp (CRSwNP) in western countries, whereas this classification was not common so far in Japan. Studying inflammatory mediators in clearly defined disease subgroups may lead to a better differentiation of chronic sinus diseases.

Methods: Homogenates of sinonasal mucosal tissue from 14 controls, 9 CRSsNP patients, and 19 CRSwNP patients were assayed for transforming growth factor (TGF)- β , interleukin (IL)-5, immunoglobulin E (IgE), *Staphylococcus* enterotoxin (SAE)-IgE, eosinophil-cationic protein (ECP), myeloperoxidase (MPO), IL-1 β , IL-6, and IL-8 by enzyme-linked immunosorbent assay or UNICAP system.

Results: CRSwNP had significantly higher levels of IL-5, IgE, SAE-IgE, and ECP compared with CRSsNP and controls. CRSsNP was characterized by high levels of TGF- β , while CRSwNP showed a Th2 polarization and lower levels of TGF- β . Especially, in CRSwNP samples, 68.4% were eosinophilic (ECP/MPO ratio >1), and 52.6% were SAE-IgE positive. On the other hand, in 9 CRSsNP patients, eosinophilic or SAE-IgE positive sample was only one sample respectively. Additionally, 31.6% of CRSwNP were asthmatic patients, while none of CRSsNP patient was suffering from bronchial asthma.

Conclusions: This study is thought to be the first one that showed the cytokine profiles in Japanese CRSs/wNP similar to those of European CRS. Based on mediator profiles, we suggest that CRSsNP and CRSwNP are distinct disease entities within the group of chronic sinus diseases.

KEY WORDS

chronic sinusitis, ECP, inflammation, nasal polyps, TGF- β

INTRODUCTION

Chronic rhinosinusitis (CRS) is one of the most common diseases in the otolaryngology area. In western countries including Europe and America, the nasal polyp is considered to be the result of eosinophilic inflammation in nasal mucosa, and it is generally accepted that CRS is classified in CRS without nasal polyp (CRSsNP) and CRS with nasal polyp (CRSwNP). Recent research has demonstrated that the pathologies of CRSsNP and CRSwNP can be differentiated into distinct subgroups on the basis of the expression of inflammatory mediators¹ and histopathology.^{2,3} On the other hand, in former Japan, CRS caused by bacterial infection held an overwhelming

majority, and the main inflammatory cell which infiltrated in tissues including nasal polyps was the neutrophil.⁴ The CRS classification such as CRSsNP or CRSwNP was not common so far in Japan. At present, however, characteristics of nasal polyp in Japan resemble those in Europe and America, and eosinophilic infiltration is a more common feature histologically.⁴ Such classification based on the pathophysiology of the disease may be useful for the decision for the treatment.

CRS is characterized by chronic inflammation of the nasal and paranasal mucosa, accompanied by tissue remodeling that includes changes in the extracellular matrix (ECM) protein deposition and tissue structure.² In western countries, CRSsNP is charac-

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Table 1 Patient characteristics and symptom scores

	Controls subjects	CRSsNP patients	CRSwNP patients	ANOVA (Fisher †)
No.	14	9	19	
Age (years; range)	62.5 (21-80)	57 (29-71)	56 (17-71)	
Female/male sex	4/10	4/5	4/15	0.216
Atopic status	0/14	2/9	7/19	0.011 †
Asthma	0/14	0/9	6/19	0.020 †
CT score	0 (0-1)	5 (2-10)	16 (10-20)	<0.001
Total symptom score	4 (3-5)	6 (2-10)	10 (4-14)	<0.001
Nasal congestion	1 (1-2)	1 (0-2)	3 (2-3)	<0.001
Sneezing	0	1 (0-1)	1 (0-2)	0.520
Rhinorrhea	0 (0-1)	1 (0-3)	1 (0-3)	0.066
Loss of smell	0	0 (0-1)	2 (0-3)	<0.001
Postnasal drip	0	1 (0-2)	1 (0-3)	0.020
Headache	0 (0-1)	2 (1-3)	1 (0-3)	0.001

The total symptom scores is the sum of the score for nasal congestion, rhinorrhea, headache, postnasal drip, loss of smell, and sneezing. The level of significance (*P*) was obtained by means of ANOVA or the Fisher exact test (marked with †); comparison between CRSsNP and CRSwNP. The CT score was obtained using the Lund-Mackay classification. The polyp size was scored according to the Davos classification.

terized by high levels of transforming growth factor (TGF)- β .^{2,3,5} In contrast, CRSwNP is characterized by a predominant Th2-biased eosinophilic inflammation with high levels of IL-5, and eosinophilic cationic protein (ECP); high levels of local IgE; and low levels of TGF- β .^{2,5,6} However, recent studies in China revealed a different inflammatory cytokine pattern with a more neutrophilic, IL-17 driven type, although remodeling pattern were similar to European patients.^{7,8} In the current study, we investigated the cytokine and mediator pattern in different subgroups of Japanese patients with CRS: CRSsNP, CRSwNP, and controls (no allergic and sinus disease). Based on previous studies in CRS with and without NP, the following groups of mediators were carefully selected: proinflammatory cytokines (IL-1 β , IL-6), eosinophil-related mediators (ECP), neutrophil-related mediators [IL-8, myeloperoxidase (MPO)], T cell and subset markers [interferon (IFN)- γ , IL-5, IL-17], a T-regulatory marker (TGF- β), and IgE [total IgE, *Staphylococcus* enterotoxin (SAE)-IgE]. The aim of this study was to possibly identify differences in the profile of inflammatory mediators and cellular characteristics for each of the selected disease subgroups in Japan.

METHODS

PATIENTS

Sinonasal mucosa from Japanese patients with CRSsNP (*n* = 9), CRSwNP (*n* = 19) and control patients (CTR; *n* = 14) was obtained at the Department of Otorhinolaryngology-Head and Neck Surgery of the Jichi Medical University Hospital, Japan. Inferior turbinates from patients without sinus disease undergoing septoplasty or rhinoseptoplasty were collected as controls based on the previous report.⁹ For

CRSsNP and CRSwNP, tissue samples originated from ethmoidal mucosa during routine endonasal sinus surgery in consecutive patients scheduled for surgery unrelated to the study. The diagnosis of sinus disease was based on history, clinical examination, nasal endoscopy, and computed tomography (CT)-scan of the paranasal cavities according to the European Position Paper on Rhinosinusitis and Nasal Polyps guidelines.¹⁰ CT scans were graded according to the Lund-MacKay method.¹¹ Individual rhinosinusitis symptoms were evaluated by a physician on a scale of 0 to 3 (no symptoms, mild symptoms, moderate symptoms, and severe symptoms) before surgery, according to the Davos classification.¹⁰ The reasons for surgical procedures were unrelated to the study in all patients. The atopic status was evaluated by using the ImmunoCAP[®] test (Phadia, Uppsala, Sweden), detecting specific IgE antibodies against House dust mite (*Dermatophagoides pteronyssinus*), Japanese cedar (*Cryptomeria japonica*), Orchard grass (*Dactylis glomerata*), Common ragweed (*Ambrosia elatior*) or Cat dander (*Felis domesticus*). The patient who showed more than class 2 for at least one of these allergens was defined as an atopic patient. The diagnosis of asthma and aspirin sensitivity was performed by a pneumologist based on lung function and challenge tests, where applicable. All patients stopped oral and topical application of corticosteroids for at least 1 month before surgery. Patients did not take any other relevant medication. An informed consent was obtained from each patient and control subject before collecting material, and the study was approved by the local ethical committee of the Jichi Medical University Hospital.

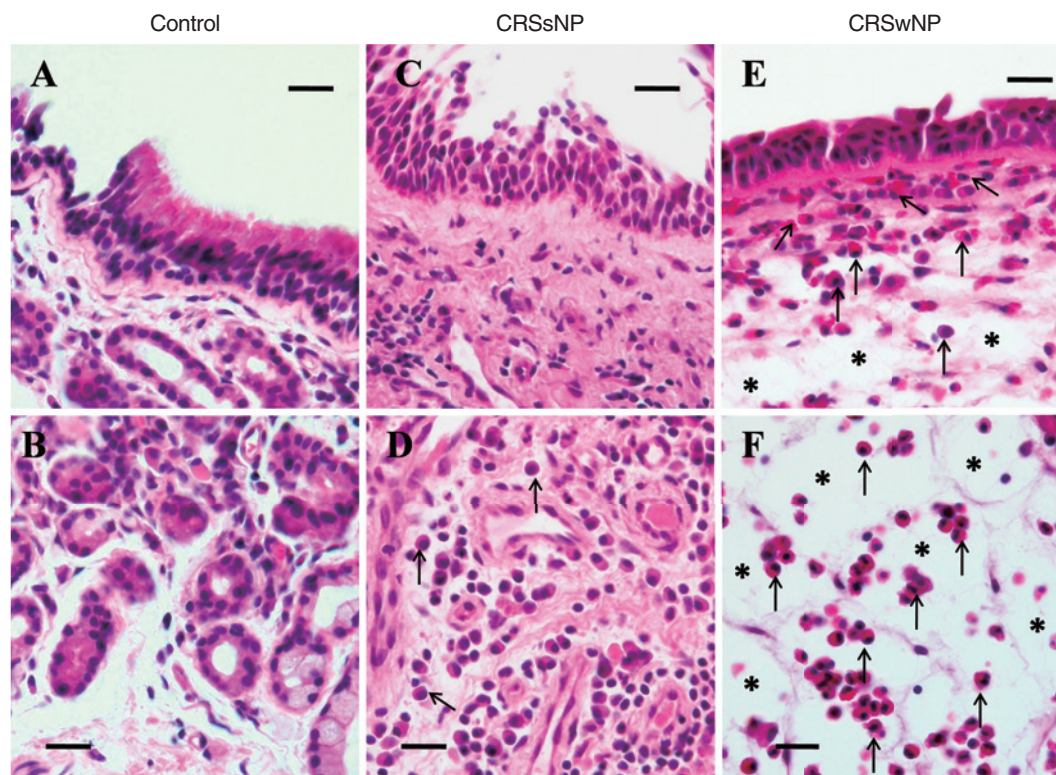


Fig. 1 Pathological findings in mucosa. The sections were stained with hematoxylin-eosin in controls (A and B), CRSsNP (C and D), and CRSwNP (E and F), respectively. Arrows indicate eosinophils. Asterisks indicate pseudocyst formation. Bars; 20 μ m.

HISTOPATHOLOGIC ANALYSIS

The tissue was fixed with 10% neutral buffered formalin solution for 1 week, embedded in paraffin, and cut into 2- μ m-thick sections. After hematoxylin-eosin (HE) staining, histopathologic analysis was performed by selecting a representative field of mucosa where the most prominent change was detected with $\times 200$ magnification. The number of eosinophils was analyzed by using a magnification $\times 200$. 5 fields of the mucosal area were evaluated at random, and the number of eosinophils/field was counted for each sample by two independent evaluators.

MEASUREMENT OF CYTOKINE AND IgE LEVELS

Freshly obtained tissue specimens were homogenized in Japan as previously described.¹² For homogenization, the tissue was thawed, weighed and 1 mL saline was added per every 0.1 g tissue. The tissue was then homogenized at 1000 rpm for 3 min on ice (homogeniser; B.Braun, Melsungen, Germany). The homogenates were centrifuged at 3000 rpm for 10 min at 4°C. After centrifugation, supernatants were aliquot in 500 μ L and stored at -80°C until analysis. All samples were assayed for IL-1 β , IL-5, IL-6, IL-8, IL-10, IL-17, IFN- γ , TGF- β and MPO by using commercially available ELISA kits (Quantikine ELISA, R&D Sys-

tems, Minneapolis, MN, USA; MPO, Oxis International, Portland, OR, USA). ECP, IgE and SAE-IgE (IgE to *Staphylococcus* enterotoxin A, C, and TSST-1; 0.35-100 kUA/L) were measured by using the UNICAP system (Pharmacia, Uppsala, Sweden). These assays were performed in Belgium following the manufacturer's instructions. An ECP/MPO ratio was calculated as described before.⁷ Standard curves and controls were performed according to the guidelines of the producer.

STATISTICAL ANALYSIS

Statistical analysis was performed with SPSS Statistics software v 17.0 (SPSS, Chicago, IL, USA). Data are expressed as median, range and interquartiles. Statistical analysis was performed by using the Kruskal-Wallis test and the Mann-Whitney *U* 2-tailed test for unpaired comparisons. When comparisons were made between groups, the Kruskal-Wallis test was used to establish the significant intergroup variability. The Mann-Whitney *U* test with with Bonferroni correction was then used for between-group comparisons. Baseline variables were analyzed by using a 1-way ANOVA test or the Fisher exact test. A statistical difference of $P < 0.05$ was considered significant.

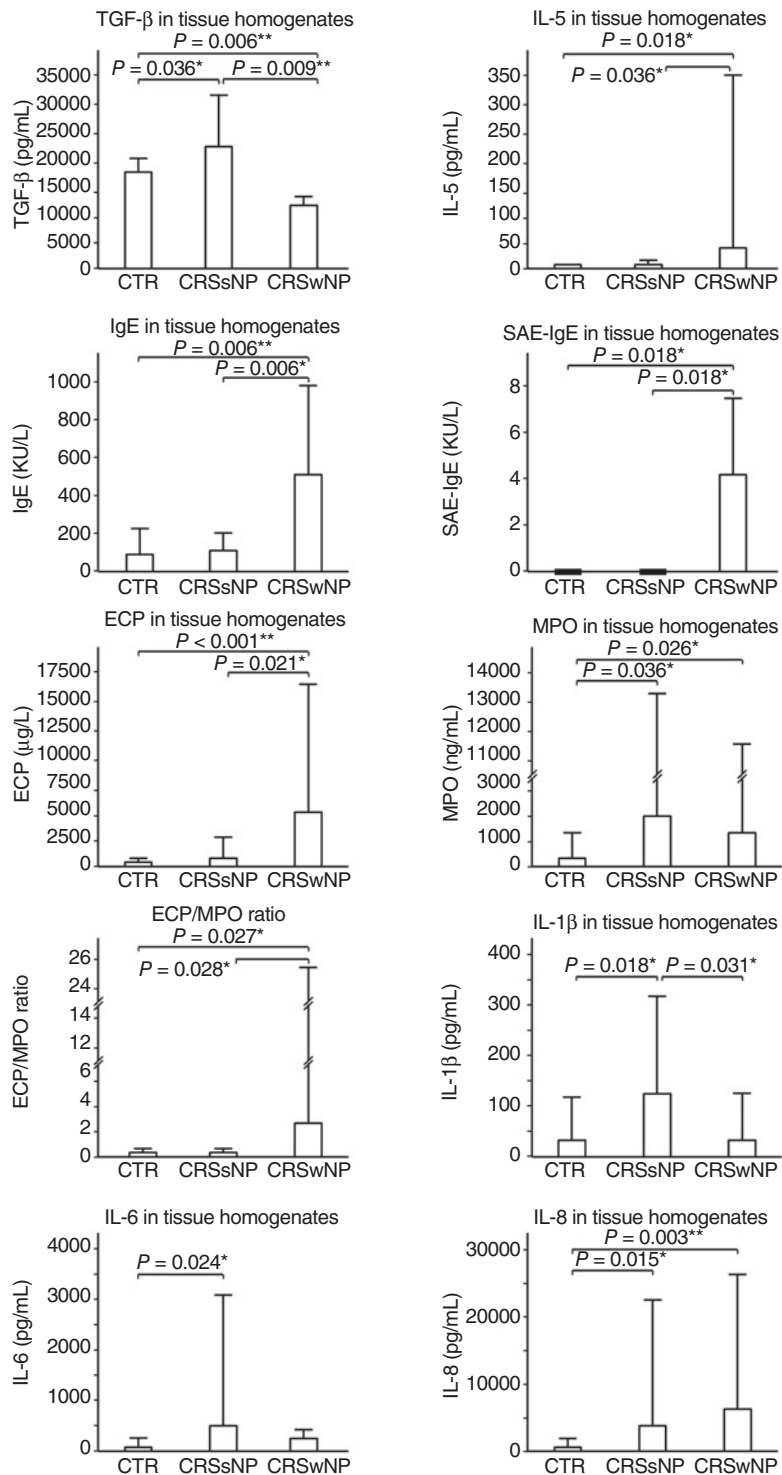


Fig. 2 Measurement of transforming growth factor (TGF)-β, interleukin (IL)-5, immunoglobulin E (IgE), *Staphylococcus* enterotoxin (SAE)-IgE, eosinophil-related mediators (ECP), myeloperoxidase (MPO), IL-1β, IL-6, and IL-8 by enzyme-linked immunosorbent assay in 14 controls (CTR), 9 chronic rhinosinusitis without nasal polyp patients (CRSsNP), and 19 chronic rhinosinusitis with nasal polyp patients (CRSwNP). Values are reported as medians and interquartile ranges for each group. Statistical analysis was performed using the Kruskal-Wallis test followed by a Mann-Whitney *U*-test with Bonferroni correction. The significance level was set at a *P* value of 0.05. From these data, an ECP/MPO ratio was calculated. **P* < 0.05; ***P* < 0.01.

Table 2 Cytokine concentrations in CRSwNP subgroups with and without atopic status

	CRSwNP with atopic status (<i>n</i> = 7)	CRSwNP without atopic status (<i>n</i> = 12)	<i>P</i> value
ECP (μg/L)	27600 (3660-58500)	5750 (2960-13100)	0.033*
MPO (ng/mL)	8875 (273-24683)	3703 (637-11558)	0.914
IL-5 (pg/mL)	511 (54-720)	26 (6-37)	0.009**
IFN-γ (pg/mL)	78 (78-78)	156 (78-1016)	0.298
IgE (KU/L)	1070 (540-1265)	354 (55-477)	0.009**
SAE-IgE (KU/L)	8.2 (5.2-14.3)	0 (0-3.8)	0.004**
IL-1β (pg/mL)	37 (23-1097)	34 (27-410)	0.762
IL-6 (pg/mL)	351 (303-1109)	95 (47-461)	0.258
IL-8 (pg/mL)	5345 (2213-25243)	8693 (1550-25708)	0.904
IL-17 (pg/mL)	11 (11-11)	11 (11-149)	0.285
TGF-β (pg/mL)	13837 (11635-18809)	13689 (9374-15239)	0.610

Data are expressed as medians and interquartile ranges. **P* < 0.05 and ***P* < 0.01.

RESULTS

PATIENT CHARACTERISTIC

Clinical characteristics and symptom scores of all patients are summarized in Table 1. A significant higher frequency of asthma was reported in CRSwNP patients, all other subjects were free of asthma symptoms. One-way anova showed that the total symptom score was the highest in CRSwNP patients of which nasal congestion and loss of smell were the most predominant symptoms. The scores for nasal congestion and loss of smell were significantly higher in CRSwNP vs CRSsNP patients, whereas CRSsNP patients reported higher symptom scores for headache compared with the other disease groups. Finally, CRSwNP showed the highest CT score compared with CRSsNP.

HISTOPATHOLOGIC ANALYSIS

HE-stained sections are shown in Figure 1. CRSwNP revealed a thickened basement membrane, a subepithelial accumulation of eosinophils and edematous change with pseudocyst formation (Fig. 1E, F). On the other hand, in CRSsNP, fibrosis and accumulation of not only eosinophils but also other inflammatory cells (neutrophils, lymphocytes and so on) were mostly found (Fig. 1C, D). The median (interquartile range [IQR]) counts for eosinophils were significantly higher in CRSwNP (15; 12-17) compared to CRSsNP (6; 4-9; *P* = 0.02) and controls (1; 0-2; *P* < 0.001).

IMMUNOASSAY (ELISA)

The information on mediators, cytokines, and IgE in tissue homogenates is shown in Figure 2. IL-5, total IgE, SAE-IgE, and ECP levels in the tissue were significantly and impressively increased in CRSwNP versus CTR and CRSsNP, whereas CRSsNP did not demonstrate any significant change in the levels of those proteins versus CTR. In clear contrast, TGF-β

protein level was significantly upregulated in CRSsNP versus CTR and CRSwNP, whereas such upregulation was not seen in CRSwNP. On the contrary, TGF-β protein level was significantly downregulated in CRSwNP versus not only CRSsNP but also CTR mucosal samples. IL-1β protein level was significantly upregulated in CRSsNP versus CTR and CRSwNP. MPO and IL-8 levels in the tissue were significantly increased in CRSsNP and CRSwNP versus CTR. IL-6 protein level was significantly increased in CRSsNP versus CTR. The ECP/MPO ratio is used to reflect the observation that eosinophils and neutrophils are present in polyps, and that the ratio between the two allows to determine which cell type is more activated. The ECP/MPO ratio was significantly increased in CRSwNP versus CRSsNP and CTR. IFN-γ and IL-17 protein data did not show any differences between groups.

In 13 samples (68.4%) of CRSwNP, the ECP/MPO ratio was more than 1, and the same samples were IL-5 positive, too. Besides, 10 samples (52.6%) of CRSwNP were SAE-IgE positive, and 9 of these 10 samples were IL-5 positive, too. On the other hand, in 9 CRSsNP patients, only 2 samples were IL-5 positive, and eosinophilic or SAE-IgE positive sample was only one sample respectively.

In CRSwNP patients, 7 of 19 were atopic, whereas only 2 of the 9 CRSsNP patients were atopic. In atopic versus non-atopic CRSwNP, ECP, IL-5, IgE and SAE-IgE levels were significantly increased (Table 2). On the other hand, in CRSwNP patients, 6 of 19 were asthmatic, and 5 of these 6 asthmatic patients were both IL-5 and SAE-IgE positive. However, none of the other group patients was asthmatic. In asthmatic versus non-asthmatic CRSwNP, ECP, total IgE, IL-8 and MPO levels were significantly increased (Table 3).

In further examination of CRSwNP, 19 CRSwNP patients could be classified in 2 groups; one was IL-5 positive group (*n* = 11), and the other was IL-5 negative group (*n* = 8).

Table 3 Cytokine concentrations in CRSwNP subgroups with and without asthma comorbidity

	CRSwNP with asthma (n = 6)	CRSwNP without asthma (n = 13)	P value
ECP (μg/L)	13150 (4410-44750)	5750 (2250-16550)	0.041*
MPO (ng/mL)	18941 (1601-24683)	637 (273-3919)	0.024*
IL-5 (pg/mL)	32 (6-720)	37 (6-65)	0.788
IFN-γ (pg/mL)	78 (78-78)	156 (78-1016)	0.298
IgE (KU/L)	975 (477-1265)	354 (143-540)	0.009**
SAE-IgE (KU/L)	7.4 (0-8.2)	0 (0-5.3)	0.316
IL-1β (pg/mL)	977 (9-1067)	29 (23-128)	0.412
IL-6 (pg/mL)	351 (76-461)	95 (47-1109)	0.412
IL-8 (pg/mL)	53400 (25243-115829)	1550 (649-5345)	0.014*
IL-17 (pg/mL)	11 (11-141)	11 (11-11)	0.495
TGF-β (pg/mL)	18809 (4089-30338)	13542 (10102-13837)	0.230

Data are expressed as medians and interquartile ranges. * $P < 0.05$ and ** $P < 0.01$.

DISCUSSION

Recent guidelines in western countries have attempted to classify CRS subgroups and unify the current pathophysiologic concepts.^{10,13} Although most of the symptoms occur in both diseases, CRSwNP have a more pronounced nasal obstruction and loss of smell, while in CRSsNP patients complain more of headache. Especially, the partial or complete loss of smell with a concomitant effect on taste is a characteristic feature of CRSwNP.¹⁴

In this study, we demonstrated clear differences in the local tissue concentrations of mediators between CRSsNP and CRSwNP. These differences are reflected by an inflammation pattern in the tissue of CRSsNP and CRSwNP, respectively. Especially, CRSwNP demonstrate increased IL-5 protein levels compared with controls and CRSsNP. Additionally, ECP as a marker of eosinophilic activation, and total IgE as another Th2 stigma are significantly up-regulated in CRSwNP versus controls and CRSsNP. Besides, in CRSwNP, these cytokine and eosinophilic mediators were increased in atopic patients versus non-atopic patients.

In western countries, ECP has been described to be significantly increased in CRSwNP vs controls and CRSsNP,^{2,15} and here we show these phenomena also found in Japanese samples. The ECP/MPO ratio was 2.4 in CRSwNP but 0.5 in CRSsNP, evidencing an eosinophil bias in CRSwNP versus a neutrophil bias of inflammation in CRSsNP. The proinflammatory mediator IL-1β and related cytokine IL-6 was significantly increased in CRSsNP vs controls. We previously reported that the pattern of cytokines in CRSwNP is remarkably different among the regions, for examples, with a Th1/Th17 dominance in South Chinese polyps and a Th2 dominance in Belgian polyps.^{7,8} The pattern of cytokines in our study is similar to that in European CRSwNP. However, there has been no study for cytokines or mediators in CRS

based on this classification in Japan. It was thought to be the first study that showed the cytokine profiles in Japanese CRSs/wNP similar to those of European CRS.

TGF-β up-regulation is characteristic for CRSsNP, whereas low TGF-β protein levels appear to be a constant finding in CRSwNP, which could point to either an increased T-regulatory cell activity¹⁶ or an increased fibrogenic potential^{17,18} in CRSsNP. An over-expression of TGF-β has been demonstrated before in CRSsNP vs CRSwNP, confirming our current results.¹⁹ TGF-β is also a potent fibrogenic cytokine that stimulates extracellular matrix formation, with increased levels contributing to fibrosis in CRSsNP, and a relative deficit in TGF-β explaining edema formation in CRSwNP. In CRSsNP, we recently reported that tissue inhibitor-1 (TIMP-1) is upregulated together with matrix metalloproteinases (MMP)-9,²⁰ and high level of TGF-β²¹ and low activity of urokinase type plasminogen activator (u-PA)²² are observed, so that fibrosis is considered to proceed in the extracellular matrix. On the other hand, in CRSwNP, TIMP-1 is not up-regulated, and high level of MMP-7/-9,²⁰ high activity of u-PA²² and low level of TGF-β²¹ are observed, so that fibrinolysis is considered to proceed in the extracellular matrix. TGF-β down-regulates u-PA by activating PAI-1 which is inhibitor of u-PA, whereas u-PA convert pro-MMPs to active MMPs via plasmin, and u-PA itself also activates MMPs.²³

It is known that SAE acting as superantigens are able to induce a polyclonal activation of T cells and cause an exacerbation of the ongoing inflammation.^{24,25} Recent insights have linked the inflammation in CRSwNP to an increased prevalence of colonization with *S. aureus* and the release of their cell products.²⁶ SAE-IgE and consecutive polyclonal IgE formation has been demonstrated in CRSwNP tissue, which correlates with markers of eosinophilic inflammation, pointing to a possible modifying role of bacte-

rial superantigens in the pathophysiology of CRSwNP.^{27,28} The presence of SAE-IgE also coincided with higher levels of IL-5, eotaxin, and ECP. The strikingly high correlation between IL-5 and IgE antibody concentrations in CRSwNP homogenates supports the hypothesis that SAE, apart from T cells, also modify B and plasma cells.¹⁵ In contrast, SAE-IgE and polyclonal IgE formation in tissue was a rare finding in CRSsNP. Besides, we demonstrated that the tissue from asthmatic or atopic patients with CRSwNP showed SAE-IgE and Th2 mediators positive with the high rate in Table 2, 3. SAE-IgE is found more frequently in NP versus controls and correlates with higher levels of IL-5 and ECP.²⁷ Moreover, an increased number of T cells expressing the TCR β -chain variable region, known to be induced by microbial superantigens, was detected in NP and correlated with the presence of SAE-IgE.²⁹ These findings confirm the role played by SAE as disease modifiers specifically in asthma and/or atopic disease with CRSwNP. Because IL-5 is thought to be highly involved in CRSwNP pathogenesis (Fig. 2), we chose it as a marker, which divides CRSwNP into subgroups. IL-5 positive CRSwNP group showed negative IL-17 and negative IFN- γ , and all asthmatic patients were included in this group. IL-5 negative CRSwNP group showed IL-17 positive, and it suggested that a certain type of CRSwNP was modified by not IL-5 but IL-17. To analyze the cytokine pattern may be important for the management of CRSwNP and the risk for the development of asthma.

An intense clinical phenotyping of patients with chronic sinus disease with the measurement of inflammatory and T-cell markers could lead to the recognition of several sinus disease entities which will allow to finally introduce new definitions for studies on the epidemiology, diagnostic management, and lower airway co-morbidities, and eventually will open differentiated and new therapeutic approaches. For example, anti-IgE, anti-IL-5 or anti-IL-17 therapy may be useful treatment for CRSwNP in the future.

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